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Characterizing the Persistence of Sewage Enterococci in Mississippi Coastal Waters

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CHARACTERIZING THE PERSISTENCE OF SEWAGE ENTEROCOCCI IN MISSISSIPPI COASTAL WATERS

by

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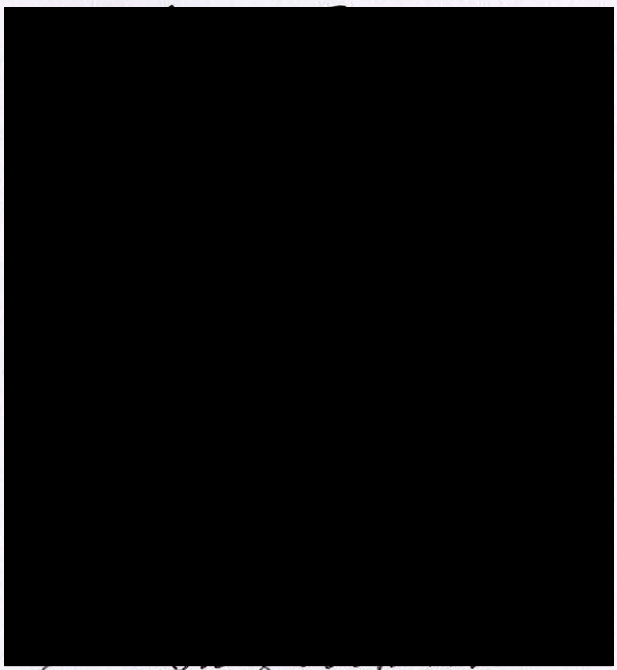

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ABSTRACT

CHARACTERIZING THE PERSISTENCE OF SEWAGE ENTEROCOCCI IN MISSISSIPPI COASTAL WATERS

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Enterococci are microbiological indicators of marine recreation water quality. Their reliability as fecal indicators is questioned as they are shown to persist in the environment. Multiple laboratory studies on their persistence have been done but few under natural environmental conditions. The purpose of this study was to investigate how long sewage enterococci and enterococcal DNA persist in beach water and to determine whether there is a difference in the genetic diversity and hardiness of sewage vs. environmental isolates. To study persistence, sewage was diluted with beach water, placed in microcosms, and deployed at a beach site in Longbeach and Pass Christian, Mississippi. Samples were analyzed for eight days using membrane filtration to enumerate enterococci, and Quantitative Polymerase Chain Reaction (qPCR) to quantify enterococcal DNA. To assess genetic diversity, BOX-PCR fingerprints of sewage and environmental isolates were compared using UPGMA cluster analysis and Simpson's diversity index. To study hardiness, growth of sewage and environmental isolates in the presence of 2.5 mM hydrogen peroxide in Enterococcosel broth were monitored for 36 hours. Results showed that after eight days, viable counts decreased on average 3.8 logs and target sequence decreased >87% in surface waters. Conversely, enterococci grew >25 folds at the bottom of the water column after four days. The diversity of sewage and environmental isolates was similar during summer months. However, the diversity of

sewage isolates declined in cooler months while that of environmental isolates remained high. The hardiness of sewage vs. environmental isolates differed as a greater portion of sewage isolates grew in the presence of hydrogen peroxide than environmental isolates. Results suggested that enterococci survival in marine water is dependent on their location in the water. Varying nutrient availability of the surface vs. bottom of the water column may be responsible for varying survival based on location. While high counts of brief duration may indicate sewage pollution, persistent high counts with no known sewage leaks may be due to re-growth or resuspension of environmental isolates. Also, consistently high genetic diversity of environmental isolates suggests an accumulation of hardier isolates over time. Their lowered resistant to hydrogen peroxide, however, indicated that oxidative damage is not their main selective agent.

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LIST OF ABBREVIATIONS

APHA	American Public Health Association
BEA	Bile esculin agar
BEACH	Beaches Environmental Assessment and Coastal Health
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
BOX-PCR	BOX-A1R-based Repetitive Extragenic Palindromic Polymerase Chain Reaction
CFU	Colony forming units
D	Simpson's diversity index
DNA	Deoxyribonucleic acid
FIB	Fecal indicator bacteria
mEI	Membrane-Enterococcus indoxyl-β-D-glucoside agar
MF	Membrane filtration
ml	Milliliter
mM	Millimolar
MPN	Most probable number
n	Number
NaCl	Sodium chloride
NTAC	National Technical Advisory Committee
p	Probability
PMA	Propidium monoazide
qPCR	Quantitative Polymerase Chain Reaction
rpm	Revolutions per minute
RO	Reverse osmosis
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
US EPA	United States Environmental Protection Agency
UV	(ultraviolet) light
VBNC	Viable but non-culturable

CHAPTER I

INTRODUCTION

Due to rising human populations along the U.S. coastline (Wilson & Fischetti, 2010), beach water quality is of increasing concern. Ensuring the microbiological safety of recreational water and water ways is a priority in the fight against illnesses related to fecal pollution. Microbial levels in recreational water need to be regularly tested in order to protect recreational users. When assessing water quality, the levels of fecal indicator bacteria (FIB), such as *Escherichia coli* and *Enterococcus spp.,* are used to indicate fecally contaminated water. These contaminated waters may contain human-specific enteric pathogens such as *Salmonella enterica* serovar Typhi, *Shigella spp.*, hepatitis A virus, and Norwalk-group viruses (Scott, Rose, Jenkins, Farrah, & Lukasik, 2002).

FIBs have certain criteria they must meet in order to be considered a good fecal indicator. Bacteria used as indicator of fecal pollution must (1) be easy to isolate and count; (2) be found in higher numbers than pathogen; (3) only be found in sewage; (4) occur where pathogens do; (5) have a density that relate to a health hazard or type of pollution; (6) have a density that relate to the degree of contamination; (7) not be able to grow in the environment; (8) be more resistant to disinfectant than the pathogens they indicate; (9) be able to be isolated from all types of water; (10) be nonpathogenic and; (11) have survival characteristics that are similar to the pathogens they indicate (Griffin, Lipp, McLaughlin, & Rose, 2001; Scott et al., 2002).

Although enterococci concentration is recommended by EPA as a criterion of beach water quality, there is increasing doubt about their reliability as an indicator of fecal pollution. Scientists question the effectiveness of enterococci and *E. coli* as FIB

because of their inability to adhere to all the criteria listed above. It appears that *E. coli* and enterococci may both persist in the environment because of available nutrients or by turning on survival mechanisms in challenging conditions and therefore might not be good FIB. Byappanahalli et al. (2006) showed that sand supplemented with lake plankton was able to support *E.coli* growth for six days. The nutrients provided by the plankton may have been responsible for the observed growth. Sand without plankton had a stable *E. coli* count with a sharp decline after five days. Yamahara, Walters, and Boehm (2009) showed that enterococci slowly grew and persisted in sediment for over 21 days. The study was done using unaltered beach sand that was intermittently wetted with seawater. Researchers found that total enterococci had a doubling time of about 1.1 days. Growth was also attributed to the organic carbon present in the water used to wet the sand or that in the sand itself. By becoming viable but non-culturable (VBNC), enterococci were also shown to survive adverse conditions such as oligotrophy, UV irradiation, and salinity changes (Heim, Lleo, Bonato, Guzman, & Canepari 2002; Lleo, Bonato, Benedetti, & Canepari, 2005). Therefore they do not adequately indicate recent pollution events.

Studies under natural conditions are needed to more accurately characterize the persistence of sewage enterococci in environmental waters. While enterococci have been shown to persist, a majority of these experiments, such as the ones in the previous paragraph, were done in mesocosms in the laboratory. The few experiments that were conducted in the environment were done in sand. For example, Byappanahalli et al. (2006) reported that both E. coli and enterococci persisted in sand at two Lake Michigan beaches during a 15 month study and were a part of the beach microflora. Enterococci persistence in sand has implications for water quality monitoring as enterococci can enter

the water during high tide, heavy rains (run-off), or a storm system where they could give a false positive signal and indicate recent pollution. When monitoring water quality, a sample of water is analyzed for FIB. However, no study has been done that examines the persistence of sewage enterococci in marine water in the natural environment. Wymer et al. (2005) demonstrated that environmental conditions are highly variable, some changing within a few minutes, hours, or daily. Therefore, using data obtained under controlled laboratory conditions may not accurately represent how sewage enterococci might survive in environmental waters.

The goal of the research was to characterize the persistence of sewage enterococci, in seawater, under natural field conditions. I hypothesize that due to differential die-off in the natural environment only hardy sewage enterococci isolates would persist, resulting in a hardier and less genetically diverse environmental population. To test this hypothesis, my specific objectives were to determine (1) how long sewage enterococci survive in beach water in coastal Mississippi; (2) whether the genetic diversity of enterococci found in sewage differed from those found in the environment and; (3) whether enterococci found in the natural environment were hardier than those found in sewage.

To study how long sewage enterococci survive in marine water, microcosms were used to deploy sewage samples at two Mississippi beach sites, one in Long Beach and the other in Pass Christian. Samples were analyzed from each microcosm and surrounding beach water for eight days using MF method (EPA Method 1600) to determine enterococci survival. As a side project, the effect of predation on enterococci survival was also determined by diluting sewage with filtered and natural beach water. EPA has

recently introduced a qPCR method (EPA Method 1611) that is faster than the traditional MF method so it was also incorporated in the study to make a comparison between the two methods.

To determine the genetic diversity of sewage and environmental enterococci populations, individual enterococci were isolated from sewage and beach water. BOX-PCR was performed on each isolate followed by capillary gel electrophoresis to visualize their DNA fingerprints. Similar banding patterns were grouped in dendrograms using UPGMA cluster analysis. Simpson's diversity index was then used to determine the genetic diversity of each population.

To evaluate the hardiness of sewage vs. environmental isolates, percent survival of sewage and environmental isolates was determined after exposure to 2.5 mM hydrogen peroxide. The same isolates used in the genetic diversity study were individually exposed to oxidative damage in Enterococcosel Broth in 96-well plates. Growth rates were inferred from optical density of individual cultures. Cultures with an optical density >1.6 was considered rigorously growing and were resistant to oxidative damage.

CHAPTER II

LITERATURE REVIEW

Development of Water Quality Standards

Water quality standards have been steadily revised over the past 100 years. One of the earliest studies was done by the American Public Health Association (APHA) in 1922. They surveyed approximately 2000 physicians using questionnaires that asked their opinions on the correlation of illnesses and bathing places (e.g. public swimming pools, beaches, etc.) (Simons, Herguson, & Gage, 1922). Based on the replies on the questionnaires, only 571 had at least one question answered with the other physicians stating they were not qualified to have an opinion in the matter. The physicians who did reply expressed that they believed there was a correlation between bathing places and infections of the eyes, ears, nose, skin, and gastro intestine.

The APHA, again in 1924, issued a report where they emphasized the importance of the development of special methods for analyzing bacterial flora for the safety of recreational users of bathing places (Simons, Gillespie, Gage, Ferguson, & Tisdale, 1924). The Joint Committee on Bathing Places was then formed and throughout the years, they tried to find evidence of the correlation between illnesses and the water quality of bathing places. In 1957, Dufour and Schaub (2007) stated that another report was issued stating that there was insufficient data to connect bathing places with the spread of disease. Due to the lack of data, by 1963, 38 states developed their own water quality standards for bathing beach waters using coliforms (ranging from 50-2400 coliforms per dL of water) and the most probable number (MPN) method to quantify coliforms, while the remaining 12 states had no water quality standards.

In 1968, the National Technical Advisory Committee (NTAC) was asked to investigate the relationship between illnesses and microbial levels in recreational waters. During their investigation, the MPN method was used which was imprecise as it overestimated the true density of coliforms and only small volumes of water could be evaluated each time (Dufour & Schaub, 2007). NTAC made the recommendation that fecal coliforms, and not just coliforms, be used as fecal indicators and that their log mean should not exceed 200 fecal coliforms/dL of water, neither should 10% of total samples collected over a 30 day period exceed 400 coliforms/ dL of water (National Technical Advisory Committee, 1968).

In 1972, the U.S. Environmental Protection Agency (USEPA) conducted a six year study in hopes of creating a bacteriological standard that could be used nationwide to make a correlation between illnesses and the microbial levels in recreational waters. They used the membrane filtration (MF) method where cellulose membrane was used to filter water samples and capture bacteria and the membrane was then placed on medium and incubated; this method was more precise than the MPN method because it could quantify the number of bacteria and larger volumes of water could be evaluated (Dufour & Schaub, 2007). The USEPA did the marine water study on three marine beaches (Boston Harbor, MA; Lake Pontchartrain, New Orleans, LA and; New York City, NY) and came to the conclusion that enterococci had the highest correlation between illnesses and microbial levels in the recreational water (Cabelli, 1983). The USEPA, in this six year study, also looked at a criterion for fresh recreational waters at Lake Erie, PA and Keystone Lake, OK. They found that the criterion developed for marine waters could not be employed for freshwaters. USEPA concluded that either *E. coli* or enterococci could

be used to make a correlation between illnesses and microbial levels in freshwater (Dufour, 1984). Based on this six year study, the USEPA recommended that enterococci, not to exceed 35 enterococci per dL of water, be used for marine recreational waters, and enterococci and E. coli, not to exceed 33 enterococci per dL of water and 126 *E. coli* per dL of water, be used for fresh recreational waters (USEPA, 1986).

By 1988, all 50 U.S. states had stopped using coliforms to monitor water quality standards based on the recommendations by NTAC in 1968 and by USEPA in 1986 (46 states were now using fecal coliforms, three states were using enterococci for marine water, and one state was using *E. coli* for freshwater) (Dufour & Schaub, 2007). In 1992 and 2003, EPA published papers which indicated that the states were slowly switching from fecal coliforms to either enterococci or *E. coli* as their fecal indicator bacteria (USEPA, 1992; USEPA, 2003). The methods of using E. coli and enterococci along with membrane filtration are still used today to monitor water quality of recreational waters.

Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000

The BEACH Act was passed in October 2000 to reduce the risk of illnesses among recreational water users. The government made it mandatory for states that had coastal recreational water to adopt new or revised criteria and standards for monitoring coastal recreational waters within three and a half years after the act was passed. The purpose of the BEACH Act was to "develop (1) an assessment of potential human health risks resulting from exposure to pathogens in coastal recreation waters, including nongastrointestinal effects; (2) appropriate and effective indicators for improving detection in a timely manner in coastal recreational waters of the presence of pathogens that are harmful to human health; (3) appropriate, accurate, expeditious, and cost-

effective methods (including predictive models) for detecting in a timely manner in coastal recreational waters the presence of pathogens that are harmful to human and; (4) guidance for state application of the criteria for pathogens and pathogen indicators to be published under section 304(a)(9) to account for the diversity of geographic and aquatic conditions" (USEPA, 2000, p. 871). The BEACH Act also required that coastal beaches be monitored for microbial levels and that the public be notified when the risk of illness from swimming is above the standard set.

Enterococci Overview

Enterococci are most often found in the feces of humans and warm-blooded animals and when they are present in water, they indicate that there is fecal pollution and possibly the presence of enteric pathogens (USEPA, 2006). Enterococci are less than 1% of the microflora of feces (Hancock & Gilmore, 2006). They are gram-positive cocci that can grow singly, in pairs, or in chains. They are facultative anaerobes and are also catalase negative. Their optimum temperature is 35°C with a temperature range of 10-45°C. Although most enterococci will grow at 35-37°C and does not require an increased carbon dioxide level, some will grow better with increased carbon dioxide levels (Facklam, Carvalho, & Teixeira, 2002). They have the ability to hydrolyze esculin in the presence of 40% bile salts, can grow in 6.5% NaCl, and can tolerate pH levels up to 9.6 (Facklam et al., 2002; Manero & Blanch, 1999). Enterococci were once considered a part of the *Streptococcus* genus due to the fact that they look the same in a Gram stain. However, it was their ability to grow in these conditions (10-45°C, pH 9.6, 6.5% NaCl) and their ability to survive heating to 60°C for 30 minutes that separated then from other *Streptococcus* (Sherman, 1937; 1938). DNA-DNA hybridization was also used to show

that *S. faecium, S. faecalis, S. casseliflavus, S. avium*, and *S. durans* had 20-50% homology (related at the genus level) when compared (Farrow, Jones, Phillips, & Collins, 1983).

Enterococci colonies have a buttery consistency, and have complex nutritional requirements (Gullberg, 1986).. Among the enterococcal species, *E. faecalis* and *E. faecium* count for most clinical isolates; *E. faecalis* accounts for about 85-90% and *E. faecium* accounts for about 5-10% (Moellering, 1992). Enterococci have been shown to be responsible for urinary tract infections (UTI), bacteremia, intraabdominal infections, and endocarditis (Hancock & Gilmore, 2006). Along with causing the above listed diseases, some enterococci have also been shown to be resistant to antibiotics like vancomycin, ampicillin, and tetracycline (Weaver, 2006) and this presents a problem when treating an enterococcal infection.

Reservoirs for Enterococci

Enterococci have reservoirs in humans, animals, and plants; *E. faecalis* and *E. faecium* are the predominant species in the human gastrointestinal tract and *E. faecium* in production animals (Fisher & Phillips, 2009). *E. casseliflavus* are associated with submerged aquatic vegetation like hydrilla (Badgley, Thomas, & Harwood, 2010). The wide range of reservoirs may bring the use of enterococci as human fecal indicator into question. However, *E. faecalis* and *E. faecium* are good focal species for the detection of human fecal pollution as they are the predominant species in human feces. Enterococci are also found in the environment because human waste from sewage and animal waste from untreated fertilizers get into the waterways and animals defecate in water (Aarestrup, Butaye, & Witte, 2002). Enterococci have also been found in soil and this can

be problematic when it rains and the soil gets washed into coastal waters through streams, thus giving a false-positive indication of fecal contamination (Fujioka, Sian-Denton, Borja, Castro, & Morphew, 1999).

Factors Influencing the Persistence of Enterococci

The concern of enterococci persisting in the environment is warranted. Enterococci persisting or multiplying in the environment would make these organisms inappropriate FIB for recreational water quality monitoring (Anderson, Whitlock, & Harwood, 2005). The persistence of enterococci is dependent on the biotic factor of predation and the abiotic factors of temperature, UV light, salinity, and oligotrophy (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012).

Predators that eliminate enterococci could be any of three types: protozoa, phages, or lytic bacteria (Barcina, Lebaron, & Vives-Rego, 1997). The persistence of enterococci in the natural environment is partially dependent on the rate of predation which is totally dependent upon the number of grazers in the water and this number will change from season to season (Boehm, Keymer, & Shellenbarger, 2005). Predation and temperature have been positively correlated (Menon, Billen, & Servais, 2003). Even though enterococci are consumed by predators, Gram negatives like *E. coli* are consumed at a faster rate because protozoa prefer feeding on Gram negatives than Gram positives (Gonzalez, Iriberri, Egea, & Barcina, 1990).

In addition to temperature and predation, UV light has also been shown to have a negative effect on enterococci survival in environmental waters (Fujioka, Hashimoto, Siwak, & Young, 1981). Direct sunlight was shown to kill indicator bacteria such as enterococci faster than indirect sunlight, as on a very cloudy day. Light from the visible

light spectrum of sunlight exhibited a bactericidal effect on fecal bacteria at a depth of at least 3.3 meters. Although sunlight inactivates bacteria, it must be noted that there are factors that will affect its bactericidal effects, such as turbidity, the chemical composition of the water, and turbulence.

Enterococci of fecal origins are considered allochthonous when released into the environment and nutrient levels they encounter in the environment might be considerably less than those present in the gastrointestinal tract where they originated. Studies show that when enterococci are exposed to nutrient poor environments, their cell surfaces become contorted after three to seven weeks and they develop resistance to multiple stressors (Hartke, Giard, Laplace, & Auffray, 1998). In these conditions they either die or enter a protective state, VBNC (Lleo et al., 2005). In oligotrophic waters, enterococci are also sometimes exposed to high salinity which also has a deleterious effect on them (Anderson et al., 2005).

Hardiness of Enterococci

Enterococci can tolerate a wide range of stressors. Rince et al (2003) showed that enterococci have adaptive responses when pretreated with sublethal doses of chemicals and physical stress factors such as pH, bile salt, and oxidative stress. Enterococci that were pre-exposed to pH 10.5 were shown to have increased tolerance to a more alkaline pH(pH 11.9) (Flahaut, Hartke, Giard, & Auffray, 1997). A pre-exposure to pH 4.8 incurred an increased survival rate of 12%, and was therefore better adapted than those at alkaline pH (0.5% survival) (Flahaut, Laplace, Frere, & Auffray, 1998). Similarly, enterococci pre-treatment with 0.08% bile salts had almost 100% survival when treated with 0.3% bile salts (Rince et al., 2003). Hydrogen peroxide (H_2O_2) exposure results in

oxidative damage to enterococci, however, tolerance to lethal concentrations of H_2O_2 (45 mM) can be induced by pre-treating the cells to sublethal concentrations of 2.0-2.8 mM for 30 minutes (Flahaut et al., 1998).

Current Methods and Media Used for the Isolation and Enumeration of Enterococci

Enterococci enumeration by MF is an EPA-approved method to evaluate recreational water samples for fecal contamination (USEPA, 2006). The water sample is filtered through a membrane filter which is then placed on Enterococcus Indoxyl-β-D-Glucoside (mEI) agar and incubated at 41°C ± 0.5°C for 24 hours. The sodium azide in mEI inhibits gram negative bacteria (Snyder & Lichstein, 1940) and indoxyl-β-D-glucoside is metabolized by β-glucosidase-positive enterococci to form the insoluble indigo blue halo. Colonies that are greater than 0.5mm in diameter with a blue halo around the outer edge are presumptively identified as enterococci. Before mEI agar, there was membrane Enterococci (mE) agar. mE agar employed the same membrane filtration method, the only difference was that the membrane had to be incubated at 41°C ± 0.5°C for 48 hours on mE agar and then transferred to Esculin Iron Agar (EIA) and incubated at 41°C ± 0.5°C for an additional 20 minutes in order to differentiate enterococci (USEPA, 2002).

Bile-esculin azide (BEA) agar is another selective medium for the isolation and enumeration of enterococci. It inhibits Gram negative bacteria and enterococci appear as black colonies due to their ability to hydrolyze esculin (Facklam et al., 2002). Gram negatives are also inhibited by the sodium azide in BEA agar. The bile that is present in BEA agar inhibits other Gram positives, but enterococci hydrolyze the esculin in the presence of bile. Before there was bile-esculin azide agar there was bile-esculin agar, but

the addition of the sodium azide made the medium more selective for Gram positive enterococci. When enterococci hydrolyzes esculin it forms 6, 7-dihydroxycoumarin which then reacts with the iron in BEA agar and forms a black precipitate which is indicative of a positive result for enterococci (Lindell & Quinn, 1975).

In addition to using selective media for the isolation and enumeration of enterococci, qPCR is also available. With selective media, only culturable cells are able grow and the weak and injured target cells might be inhibited by the selective agents. qPCR analyzes all target cells (live and dead) (Santo Domingo, Siefring, & Haugland, 2003). The current qPCR method published by U.S. EPA has not yet been approved. It amplifies the large submit ribosomal ribonucleic acid (lsrRNA) gene, 23S rRNA, of enterococci (USEPA, 2012). A sample of water is collected, filtered, and salmon testes DNA is added to each sample and is used as the sample processing control (SPC) to correct for differences in DNA recovery and to indicate PCR inhibition. DNA is then extracted using a crude method of homogenizing then clarifying by centrifugation. Enterococcal DNA is amplified using TaqMan® PCR Master Mix and probe system. DNA is then quantified using the comparative cycle threshold (C_T) method to determine the absolute quantity of lsrRNA gene copies (target sequence) in the water sample. The C_T method calculates the ratio of target sequence in water samples, relative to target in calibrator samples of known enterococci concentration. This allows the calculation of calibrator cell equivalents of water samples. The advantage of this identification method is its rapidity. Results are available three to four hours after processing and permits same day notification to the public of recreational water quality (USEPA, 2012). Similar

methods were previous developed (Haugland, Siefring, Wymer, Brenner, & Dufour, 2005; Ludwig & Schleifer, 2000).

CHAPTER III

MATERIALS AND METHODS

Microcosms

Microcosms that can be deployed in coastal beach waters under natural conditions were used. The microcosms were made by the fabrication shop in the Shelby Freland Thames Polymer Science Research Center on the campus of The University of Southern Mississippi. The body of each microcosm was fabricated from Delrin plastic and measured 39 x 63 x 63 mm (L x W x H). Each microcosm held 45 ml of liquid sample and was sealed on two opposite sides using 47 mm circular polycarbonate (PC) membrane of 0.2 μm pore size and silicone. The membranes allowed dissolved substances in the surrounding water to diffuse in and out of the microcosm but prevented entry or exit of bacteria and protozoan. A nylon mesh covered with a stiff polyethylene screen was affixed to the microcosm to prevent damage to the membrane by current surges and puncture by objects in the water. Liquid samples were added and removed through a silicone septum on one side of the microcosm. A brick was used as anchor and closed-cell foam was used as the float. A three foot rope was used to link the float and the anchor via eyebolts that were anchored in both the float and anchor. Microcosms were then zip-tied onto the eyebolt in the float. An identification and *Research in progress* tag was also attached to the anchor.

Sample Collection and Enterococci Isolation or Enumeration

Enterococci were isolated from both sewage and beach water. Sewage was obtained from either the lift station east of McCarty Hall on the campus of the University of Southern Mississippi or the lift station on Westover Drive, Hattiesburg, Mississippi.

Beach water was obtained from either a beach site in Gulfport (30°22'8.00"N, 89° 4'47.45"W), Long Beach (30°19'57.58"N, 89°10'48.55"W), or Pass Christian (30°19'40.01"N, 89°12'5.01"W) , Mississippi. Sewage and environmental samples were transported in autoclaved one L containers to the laboratory or beach site on ice and used within six hours of collection.

To isolate enterococci, sewage was diluted with 15 ml sterile 1X Phosphate Buffered Saline (PBS), filtered through 0.45 um gridded mixed cellulose esters membranes (Pall Corporation, Ann Arbor, Michigan) and plated on mEI. Enterococci concentration in sewage varied, ranging from 3.6×10^2 CFU/ml to 3.6×10^3 CFU/ml with an average of 2.0×10^3 CFU/ml. Beach water was simply filtered and membranes also plated on mEI. All mEI plates were incubated at 41°C ± 0.5°C for 24 hours. Colonies with a blue halo and ≥0.5 mm in diameter were presumptively identified as enterococci in accordance with EPA Method 1600 (USEPA, 2006).

Confirmation of Enterococci Isolates

Presumptively identified enterococci were confirmed on the basis of their heat and salt tolerance, and ability to hydrolyze esculin. Isolates obtained from mEI plates were grown on Brain Heart Infusion agar (BHIA) (EMD Chemicals Inc, Gibbstown, New Jersey) at 45°C for 24 hours. Subsequent isolates were then grown on BHIA infused with 6.5% sodium chloride at 37°C for 48 hours. Isolates that grew in the presence of 6.5% salt were then grown on Bile Esculin agar (BEA) (Hardy Diagnostics, Santa Maria, California) at 37°C for 24 hours.

QPCR

To extract enterococcal DNA, one ml of sample was filtered through a 0.4 um polycarbonate membrane with a 13 mm diameter using a Swinnex filter holder (EMD Millipore Corporation, Billerica, MA) and a sterile one ml syringe. The filter was then placed in a sterile 1.5 ml extraction tube with 0.3 ± 0.01 g 0.5 mm zirconia or silica beads (BioSpec Products, Inc., Bartlesville, OK) and Salmon DNA/extraction buffer (USEPA, 2012). The samples in the tubes were homogenized for 20 seconds with a Precellys® 24 Lysis and Homogenization Automated Equipment (Bertin Technologies, Rockville, MD). Homogenate was then clarified by centrifugation at 12, 000 rpm for one minute and five minutes to obtain crudely extracted DNA. DNA was also extracted from *Enterococcus faecalis* ATCC 29212 using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, CA) for the DNA standards.

Enterococcal DNA was detected with qPCR using primers EntEPA-F (5'- GAG AAA TTC CAA ACG AAC TTG- 3') and EntEPA-R (5'- CAG TGC TCT ACC TCC ATC ATT- 3') and probe EntEPA-P (5'- FAM-TGG TTC TCT CCG AAA TAG CTT TAG GGC TA-TAMRA -3') (Ludwig & Schleifer, 2000). The PCR master mix contained one uM of each primer, 80 nM of the probe, 1X EconoTaq® Plus Master Mix (Lucigen, Middleton, Wisconsin) and five ul enterococcal DNA for a total volume of 25 ul. QPCR cycling parameters included two holding steps of 50°C for two minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, and 60°C for two minutes (EPA Method 1611). Fluorescence signal was detected after each cycle. QPCR was carried out using an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

DNA was then quantified using the comparative cycle threshold (C_T) method to determine the absolute quantity of lsrRNA gene copies (target sequence) in the water sample. The ratio of target sequence in water samples, relative to target sequence in calibrator samples of known enterococci concentration was calculated. From this ratio, calibrator cell equivalents of water samples can now be calculated (USEPA, 2012).

Field Experiments on Persistence of Sewage Enterococci

Field studies of sewage enterococci persistence were carried out at two public beaches in Long Beach and Pass Christian (GPS coordinates above) in June, July, August, and October 2012 and January 2013. A total of eight microcosms were used each time at each site. Sewage was first mixed with filtered or natural beach water at a ratio of 1:1 and then added to the microcosms using a sterile 60 ml syringe. Sewage diluted with filtered beach water was placed in four microcosms and sewage diluted with natural beach water placed in the remaining four microcosms. The microcosms and the surrounding beach water were sampled at regular intervals to determine viable enterococci concentration and enterococcal DNA concentration. Samples were taken on Days 0, 1, 2, 3, 4, and 8 except for those from the June study which were sampled on Days 0, 2, 4, and 8. In general, 1.5, 2, 2.5, 3, and 45 ml were sampled on Days 0 and 1, 2, 3, 4, and 8, respectively. To obtain sufficient enterococci for reliable enumeration, actual sample volumes were adjusted slightly with each experiment depending on the initial sewage enterococci concentration. For surrounding beach water, 250 ml was sampled each time. Samples were transported back to the laboratory on ice in sterile tubes or bottles and processed within six hours of collection.

DNA was extracted from one milliliter of each sample and assayed using qPCR for the molecular detection of enterococcal DNA as described above. Viable enterococci were enumerated from the remaining sample as described above. Colonies were counted, adjusted for dilution factor, and enterococci concentration was expressed as $\log_{10}$ CFU/dL. The temperature, salinity, and turbidity of the beach water were also measured. Microcosm samples were brought back to the lab on the eight day. Isolates obtained on mEI were used for hardiness and genetic analyses. These isolates were referred to as Day-8 sewage isolates.

Laboratory Experiments on Persistence of Sewage Enterococci

Laboratory studies of enterococci persistence were carried out using 125 ml flasks instead of microcosms. Sewage was first diluted 1:1 with either filtered or natural beach water adjusted to 22 ppt with artificial sea salt in a beaker. Fifty milliliter was then poured into each of four autoclaved 125 ml flasks. The flasks were placed in an incubator that cycled between 28°C and 35°C daily to simulate diurnal fluctuations in water temperature at a beach during the summer. A 14:10 light: dark photoperiod was maintained in the incubator using a 96 watt fluorescent power compact light fixture. The flasks were shaken at 150 rpm. The flasks were sampled using the same sampling schedule as the field study and viable enterococci enumerated using EPA Method 1600.

To determine the effect of cell density on enterococci predation, an experiment was carried out using sewage diluted 1:1 and 1:100 with natural beach water. Triplicate flasks were used for each dilution at each site for a total of six with beach water from Gulfport, six with beach water from Long Beach, and six with beach water from Pass Christian. The flasks were shaken at 85 rpm. The 1:1 dilutions were sampled daily for

four days and the 1:100 dilutions were sampled on Days 0 and 4. Viable enterococci were enumerated as described above.

Effect of Water Column Location on Persistence

A one L sewage sample was collected and transported to the Pass Christian site on ice. Sewage was diluted 1:1 with filtered beach water and 45 ml was placed in each of 20 microcosms with 0.2 um membrane glued in place. Ten microcosms were attached to the bottom of the floatation device (upper water column) and ten to the anchor (lower water column) and deployed in three feet of water. Microcosms were sampled on Days 0 and 4 to determine enterococci concentration.

BOX-PCR

BOX-PCR was used for DNA fingerprinting of enterococci isolates for genetic analysis. Enterococci were isolated from sewage (n = 227), beach water (n = 228), and Day-8 samples (n = 210) during summer (June-August 2012) and fall (October 2012) and confirmed as described above. To prepare isolates for testing, confirmed enterococci were used to inoculate 500 ul BHIB in 96-well deep well plates. Overnight cultures were washed twice in 500 ul RO water and resuspended in 50 ul sterile RO water. The washed cells were used as the library for both BOX-PCR and to inoculate overnight cultures for the hardiness assay. For BOX-PCR, one microliter of resuspended cells was used in each amplification reaction. Each PCR reaction had a final volume of 10 ul, including the one ul of enterococci cell suspension.

The PCR master mix contained two μM primer (BOX A 1R [5'- CTA CGG CAA GGC GAC GCT GAC G- 3']) and 1X Takara PrimeSTAR® GXL DNA Polymerase (Takara Bio Inc., Japan) composed of 1X PrimeSTAR GXL Buffer, 200 uM dNTP, and

0.25 units of PrimeSTAR® GXL DNA Polymerase. PCR cycling parameters included an initial denaturation at 95°C for two minutes, 35 cycles of 94°C for three seconds, 92°C for 30 seconds, 50°C for 60 seconds and 65°C for eight minutes followed by a final extension step at 65°C for eight minutes. PCR products were diluted 1:10 with RNase/DNase free water (Teknova, Hollister, CA) and visualized using a QIAxcel Advanced System capillary electrophoresis system. QX Alignment Marker of 15 bp and 15 kb were added to all samples for normalization of lanes.

Determination of Genetic Diversity

To determine the genetic diversity of sewage vs. enterococci isolates, BOX-PCR was used to amplify enterococcal DNA from sewage, environmental, and Day-8 sewage isolates as described above. The fingerprint patterns were analyzed by BioNumerics version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) bioinformatics software. Genetically identical isolates were identified from dendrograms created using the unweighted pair group method with arithmetic mean (UPGMA) for cluster analysis of Dice Similarity Co-efficient. Pattern optimization (shift allowed between two patterns that give optimum alignment for best possible match) was set to 0.5%, and band tolerance (maximum shift allowed between two bands for them to be considered matching) was set to 0.75% with a 0.5% gradual tolerance increase towards to bottom of the gel. Genetic diversity was assessed by analyzing richness (number of fingerprints) using the Simpson's (D) diversity index ($D = 1/\sum_{i=1}^{S} Pi \text{ ^} 2$), where S = total number of species in the community and P_i = proportion of S made up of the i^{th} species.

Hardiness of Sewage and Environmental Isolates

The same enterococci isolates used in the genetic diversity study were in the hardiness assay. A 96-pin MULTI-BLOT Replicator (V&P Scientific, Inc., San Diego, California) was used to transfer one ul of enterococci cell suspension from the library to 100 ul BHIB in untreated 96-well cell culture plates (Sarstedt, Newton, N.C) and incubated overnight at 37°C.

To test the hardiness of isolates, one ul of each overnight was used to inoculate 150 ul BBL Enterococcosel Broth (Becton Dickinson Microbiology Systems, Sparks, Maryland) in untreated 96-well cell culture plates. The broth contained 2.5 mM H_2O_2, made from a 30% H_2O_2 in water stock (Fisher Scientific, Pittsburgh, PA). The isolates were also inoculated in Enterococcosel Broth without H_2O_2 as positive controls. Each plate had six un-inoculated wells that served as negative controls to monitor for contamination. All plates were incubated at 30°C to simulate environmental water temperature during the summer. Growth was monitored every 12 hours for 36 hours by optical density at 480 nm. Plates were read immediately after inoculation and the average was calculated for all the negative control wells. All data were then normalized by subtracting the averaged initial blank. An optical density reading $\geq$1.6 indicated rigorous growth and resistance to oxidative damage.

Data Analysis

An unpaired, two tailed t-test was used to assess the relationship between (1) filtered and natural beach water samples in persistence study; (2) the 1:1 and 1:100 dilutions and; (3) enterococci survival in the lower and upper water column. Differences

with $\alpha \leq 0.05$ were considered statistically significant. Linear regressions were calculated using SigmaPlot v.9.0.

CHAPTER IV

RESULTS

Persistence of Sewage Enterococci

Sewage enterococci do not appear to persist in the water column as their concentration quickly declined over time. Enterococci counts dropped an average of 0.8 logs with a range of 0.1-1.6 logs after two days in environmental waters, at both sites. By the fourth day, counts declined further by an average of 2.1 logs with a range of 0.7-3.5 logs. Enterococci counts had declined even further after eight days by an average of 3.8 logs and a range of 2-5.2 logs (Figures 1A, 1B, 2A, 2B, 3A, and 3B). Amongst initial enterococci concentration, <0.5% were still viable on the eighth day.

Parallel results were observed when the field study was repeated in the laboratory. Under controlled laboratory conditions, enterococci counts declined 0.5, 2.6, and 3.7 logs on the second, fourth, and eighth day, respectively (Figure 4). These results confirmed the results observed in the field study above.

Similarly, enterococcal DNA does not persist in the water column as their concentration also quickly declined. After two days, initial DNA concentration had decreased an average of 30% with a range of 30-76%. DNA concentration declined even further to an average of 84% with a range of 55-98%. On the eighth day of field studies, DNA concentration declined an average of 88% with a range of 86-99% (Figures 5A, 5B, 6A, and 6B). These results support the viable counts data above and indicate that enterococci die over time.

Sewage enterococci decline overtime regardless of season but the rate of decline was greater during summer months than in fall or winter months. In summer months,

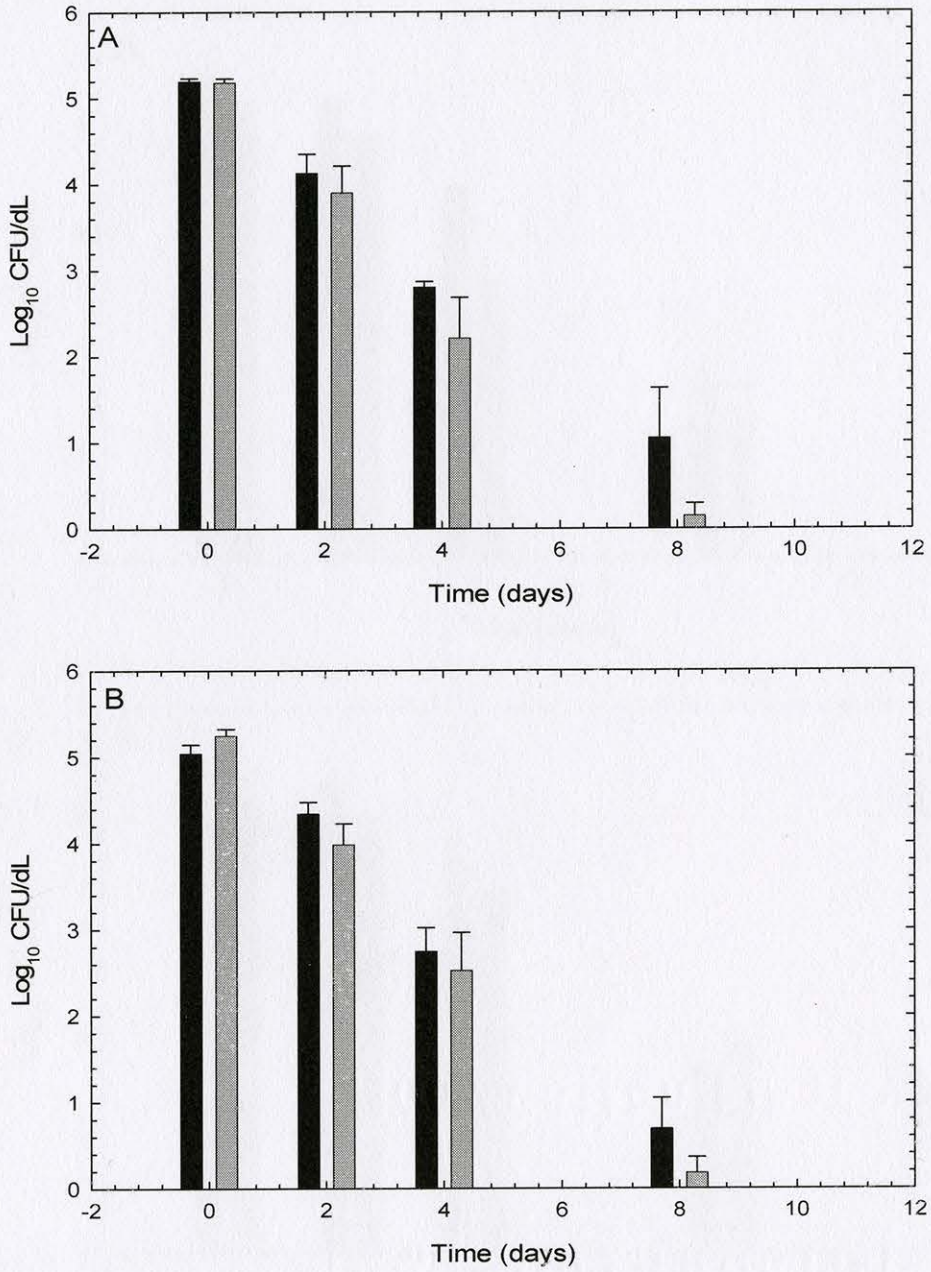

Figure 1. Survival of sewage enterococci during summer 2012 at a public beach in Long Beach (A) and Pass Christian (B). Black bars are filtered beach water and sewage and grey bars are natural beach water and sewage. Error bars are the averaged standard errors of four replicates. Graphs are the average of the June, July, and August field studies.

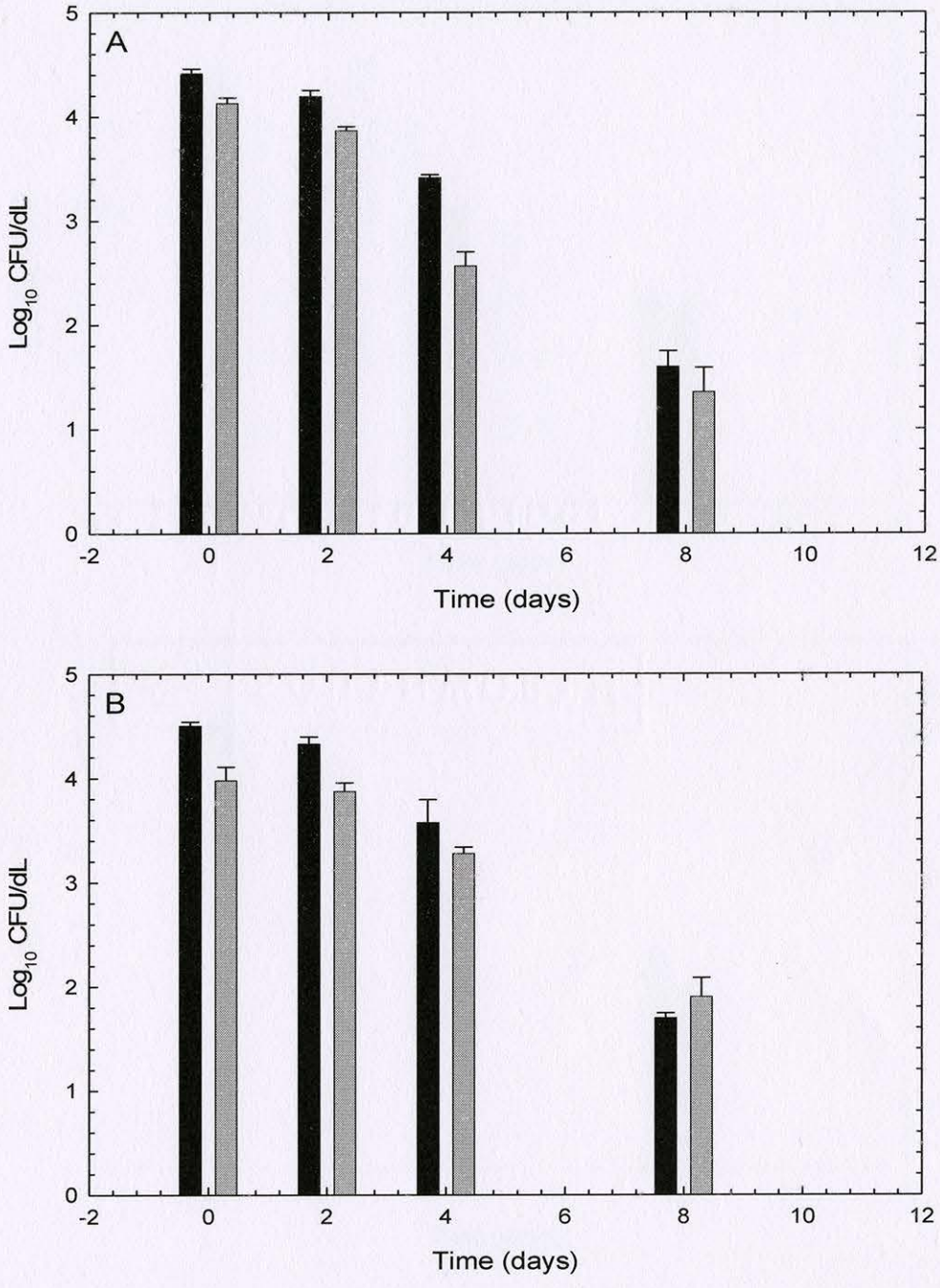

Figure 2. Survival of sewage enterococci in October 2012 at a public beach in Long Beach (A) and Pass Christian (B). Black bars are filtered beach water and sewage and grey bars are natural beach water and sewage. Error bars are standard errors of four replicates.

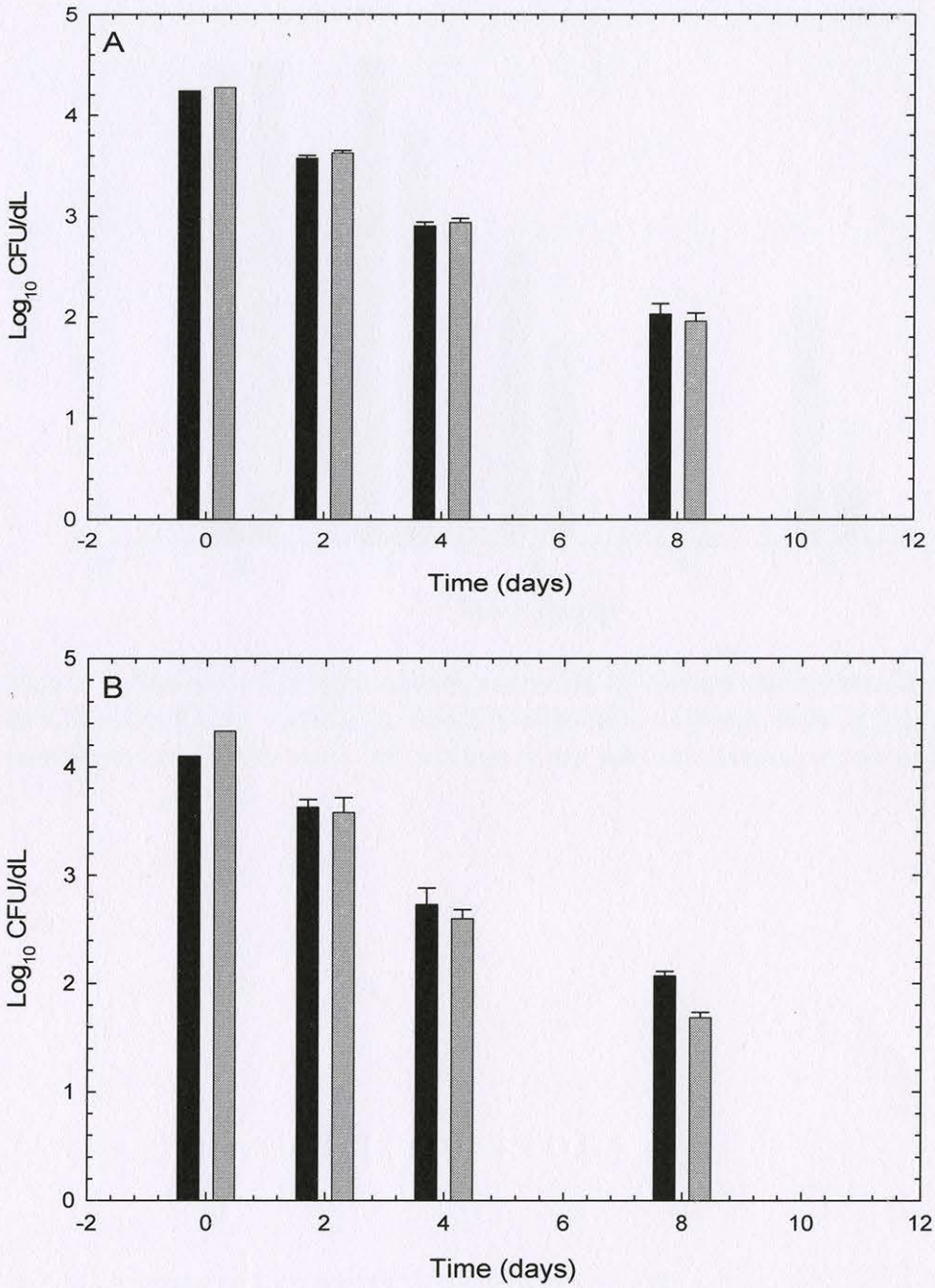

Figure 3. Survival of sewage enterococci in January 2013 at a public beach in Long Beach (A) and Pass Christian (B). Black bars are filtered beach water and sewage and grey bars are natural beach water and sewage. Error bars standard errors of four replicates.

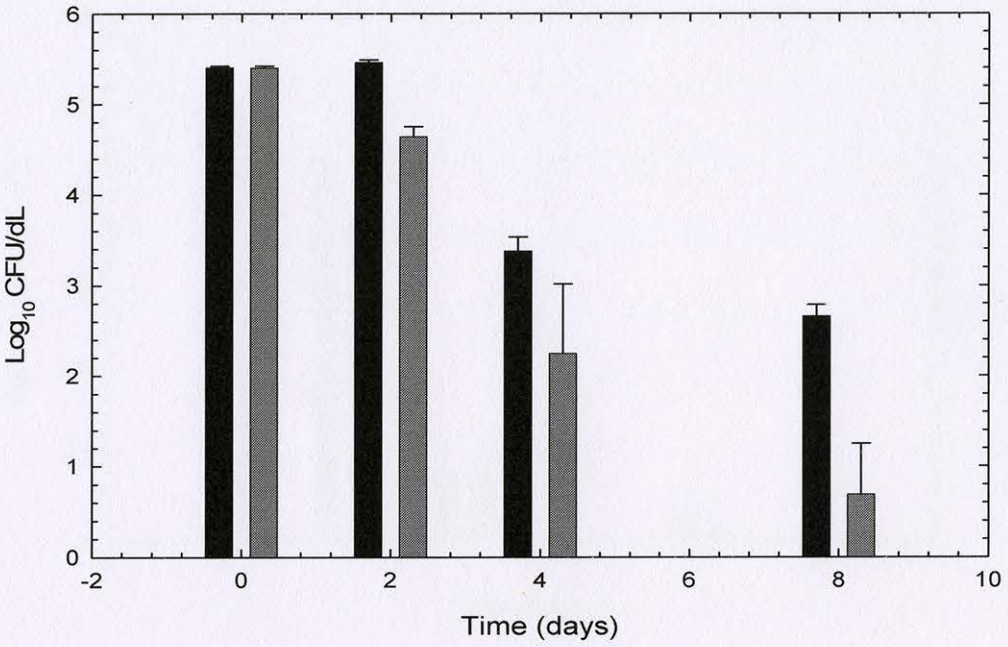

Figure 4. Survival of sewage enterococci in the laboratory under simulated environmental field conditions. Black bars are filtered beach water and sewage and grey bars are natural beach water and sewage. Error bars are standard errors of four replicates.

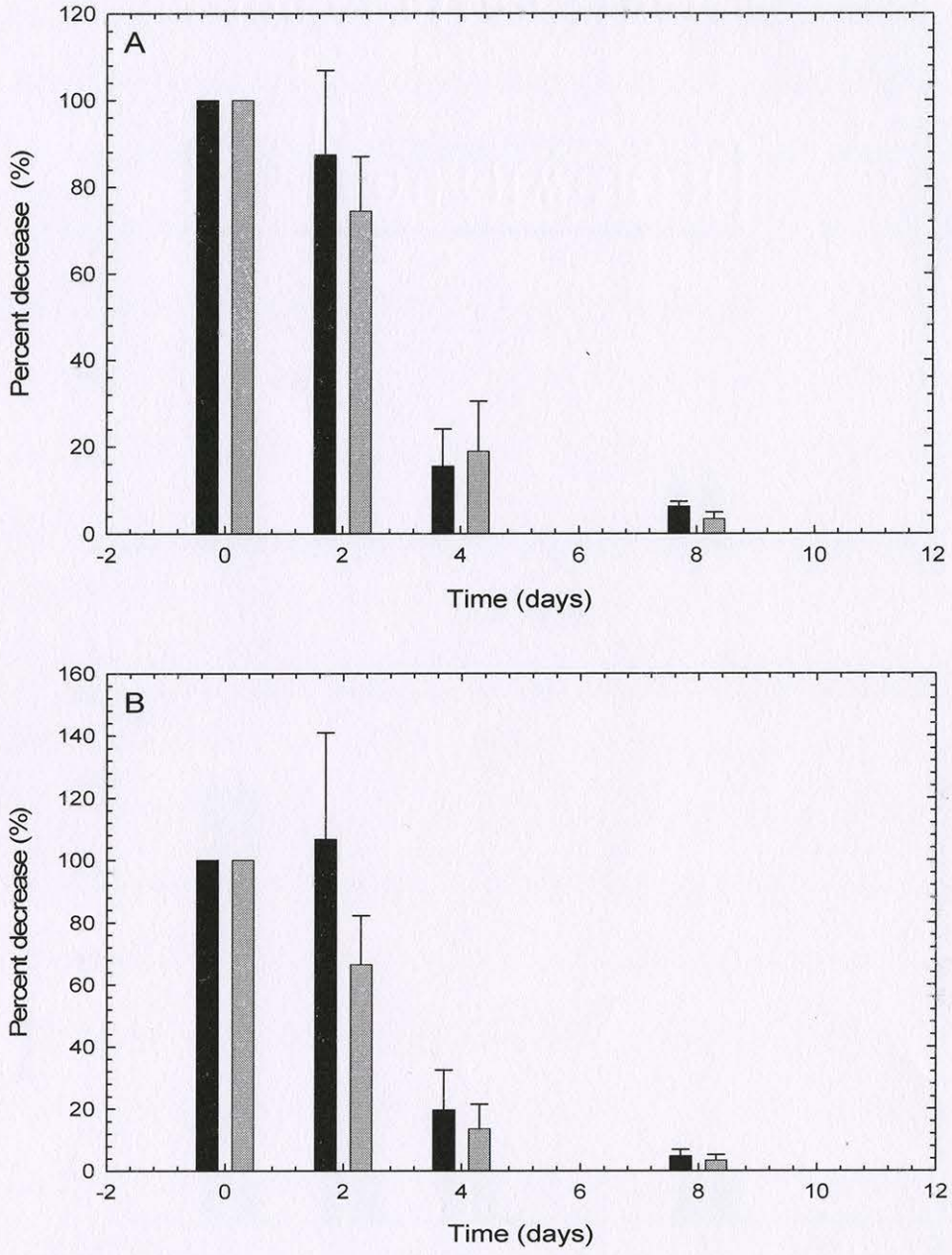

Figure 5. Percent decrease of sewage enterococcal DNA during summer 2012 at a public beach in Long Beach (A) and Pass Christian (B). Black bars are filtered beach water and sewage and grey bars are natural beach water and sewage. Error bars are the averaged standard errors of four replicates. Graphs are the average of the June, July, and August field studies.

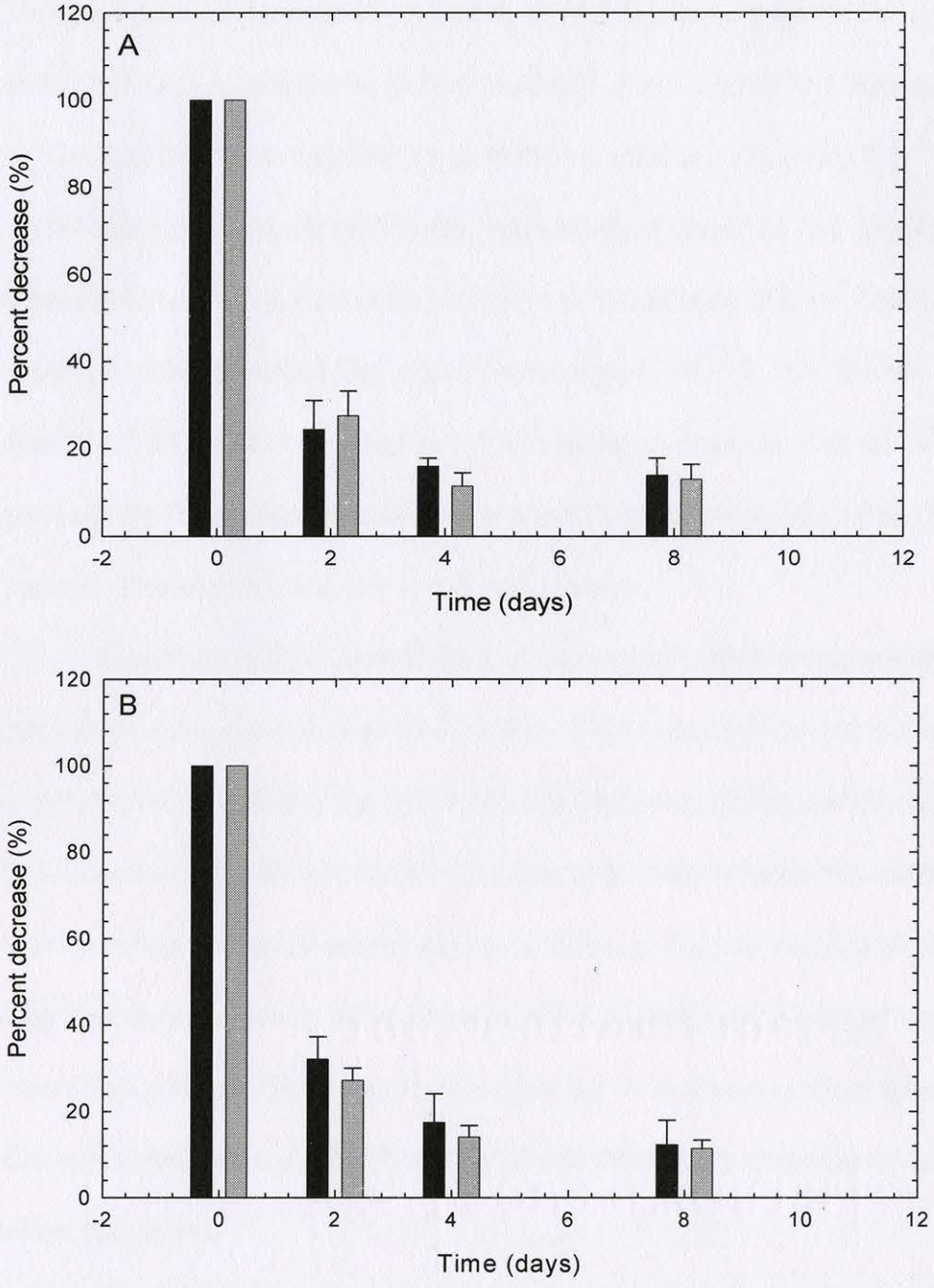

Figure 6. Percent decrease of sewage enterococcal DNA in October 2012 at a public beach in Long Beach (A) and Pass Christian (B). Black bars are filtered beach water and sewage and grey bars are natural beach water and sewage. Error bars are standard errors of four replicates.

viable enterococci decreased by 1.1, 2.6, and 4.7 logs on average on the second, fourth, and eighth days, respectively, of field studies (Figures 1A and 1B). However, the rate of decline was less in the fall than it was in the summer, declining only 0.2, 1.1, and 2.6 logs on the second, fourth, and eighth day, respectively (Figures 2A and 2B). Likewise, the rate of decline was also less during the winter, declining by 0.7, 1.5, and 2.3 logs on the second, fourth, and eighth day, respectively (Figures 3A and 3B). The water temperature was $30 \pm 2.2^{o}C$, $25 \pm 0.4^{o}C$, and $16 \pm 0.4^{o}C$ during the summer, fall, and winter, respectively. The progressive decline in water temperature could explain the increased survival of sewage enterococci in the water column.

Enterococcal DNA rate of decline also seemed dependent upon season. During summer months, enterococcal DNA declined 17, 83, and 96% on the second, fourth, and eighth day, respectively (Figures 5A and 5B). However, during the fall, enterococcal DNA decreased 73, 85, and 88% in the same time frame (Figures 6A and 6B). The decrease observed on the second day in the summer was less than that of the fall which may have been caused by the variation in DNA concentration observed on Day 2 at both sites (Figure 5A and 5B). Note however that the DNA decrease observed on the eighth day was higher in the summer than the fall and this trend was similar to that of the viable count data above.

Predation appeared to be one of the factors responsible for sewage enterococci decline in the water column. Sewage diluted 1:1 and 1:100 declined one and two logs, respectively, in enterococci concentration (Figure 7C). These results indicate that the effect of predation was masked by the high enterococci concentration in the microcosms which resulted in a low predator: prey ratio. The masking of the predation effect explains

the variation observed when the effect of predation was assessed using a Student's t-test to compare enterococci decline between filtered and natural beach water samples (Table 1). However, the effect of predation appeared to be similar regardless of where the natural beach water used to dilute the sewage came from. For example, after four days the 1:1 dilution with natural beach water from Gulfport (GP), Long Beach (LB), and Pass Christian (PC) had a decrease of 0.9, 1.2, and 0.9 logs, respectively (Figure 7A). Likewise, the 1:100 dilutions had a decrease of 2.1, 2, and 1.9 logs for GP, LB, and GP, respectively (Figure 7B).

Enterococci survival in environmental waters seems to depend on their location in the water column. After four days, enterococci in the upper water column had an average decrease of 1.7 logs, which was >45 fold decrease. Those in the lower of the water column, however, had a 1.4 log increase, which was >25 fold increase (Figure 8). These results seem indicative of a nutrient gradient in the water column.

A regression analysis of the results obtained with MF method and qPCR revealed a positive correlation (r^2, 0.39). However, the degree of the correlation was weak. The slope was >2 indicating that while DNA increases as cell number increases the rate of DNA increase is more rapid (Figure 9).

Determination of Genetic Diversity

The genetic diversity remained high among enterococci in beach water regardless of season but varied depending on season among enterococci in sewage. In the summer the Simpson's diversity index indicated a high genetic diversity for the environmental and sewage population, D = 68.53 and 72.98, respectively (Figure 10A). In the fall, the genetic diversity of the environmental population remained high, D = 65.63. The sewage

population, on the other hand, showed a dramatic decrease in the fall compared to that of the summer, D = 7.13 (Figure 10B).

Sewage enterococci that remained in the microcosms after eight days had greatly reduced genetic diversity compared to initial sewage enterococci, thus showing selection. In the summer, the Simpson's diversity index showed that the diversity of the sewage isolates (D = 72.98) was much higher than that of the Day-8 sewage isolates (D = 26.65) (Figure 11A). In the fall, the same trend was observed for the sewage and Day-8 sewage population diversity (D = 7.13 and 3.30, respectively) (Figure 11B). These results indicate selection in environmental waters. Figure 12 is an example of the dendrograms used to compare fingerprints for genetic diversity study.

Table 1

P-values of the Student's t-test showing differences in enterococci survival between filtered and natural beach water at the Long Beach and Pass Christian sites after eight days

	Viable Counts		qPCR Quantification	
Experiments	Long Beach	Pass Christian	Long Beach	Pass Christian
June 2012	0.087	0.28	0.68	0.54
July 2012	0.0019*	0.089	0.074	0.14
August 2012	0.0051*	0.0019*	0.52	0.77
October 2012	0.87	0.0091*	0.16	0.52
January 2013	0.47	0.00035*	ND	ND
Laboratory†	0.013*	-	-	-

*statistical significance

ND (no data)

†laboratory study only and not at any field sites (only viable counts)

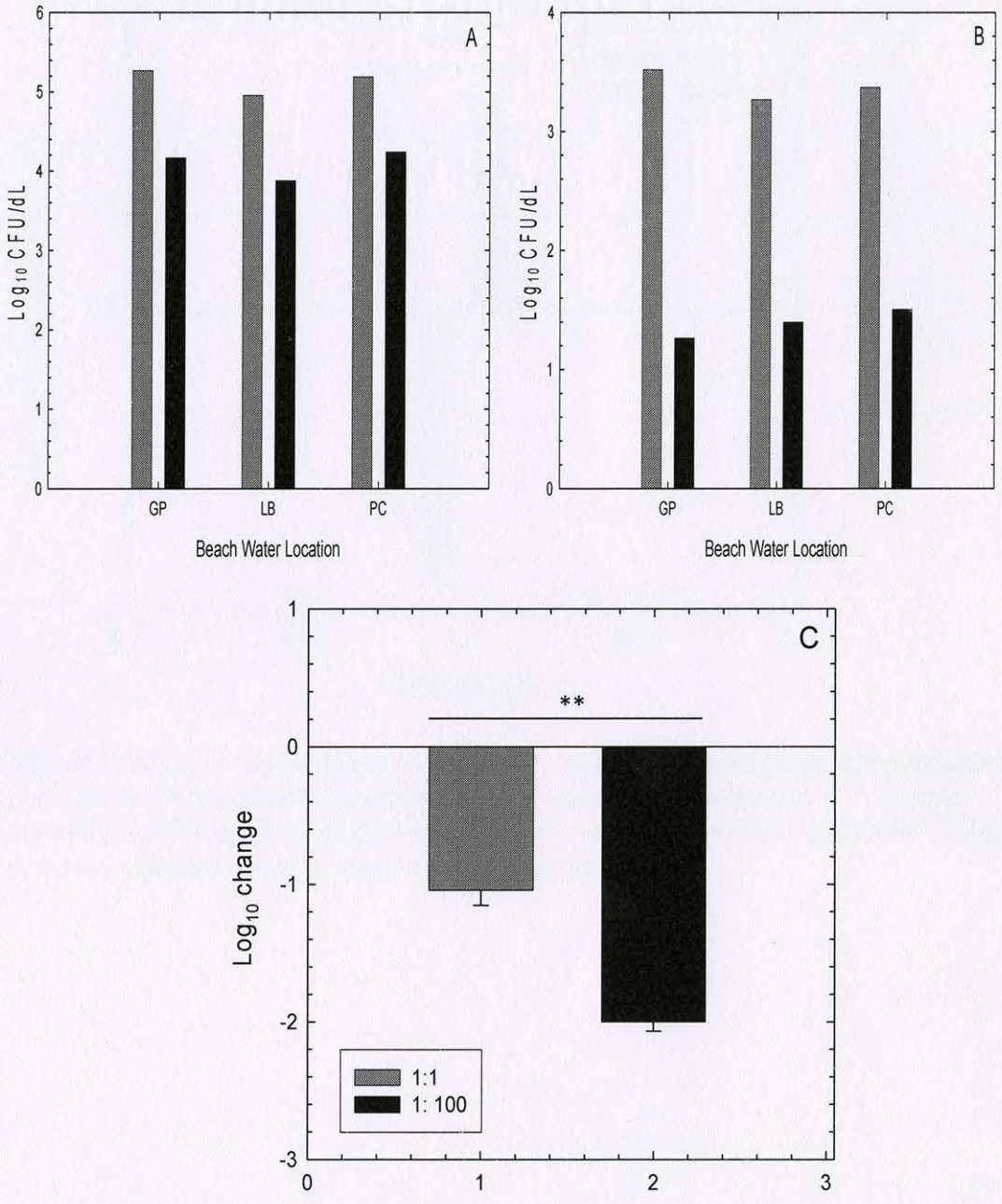

Figure 7. Effect of enterococci cell density on the effect of predation. (A) 1:1 dilution of sewage and beach water. (B) 1:100 dilution of sewage and beach water. C) The averaged Log_{10} change in enterococci concentration after four days of incubation. In A and B, grey bars represent initial enterococci concentration and black bars represent enterococci concentration after four days of incubation. In C, error bars represent standard error of all like samples. ** denotes significant differences between the two dilutions with a P-value = 0.00184. GP = Gulfport, LB = Long Beach, and PC = Pass Christian.

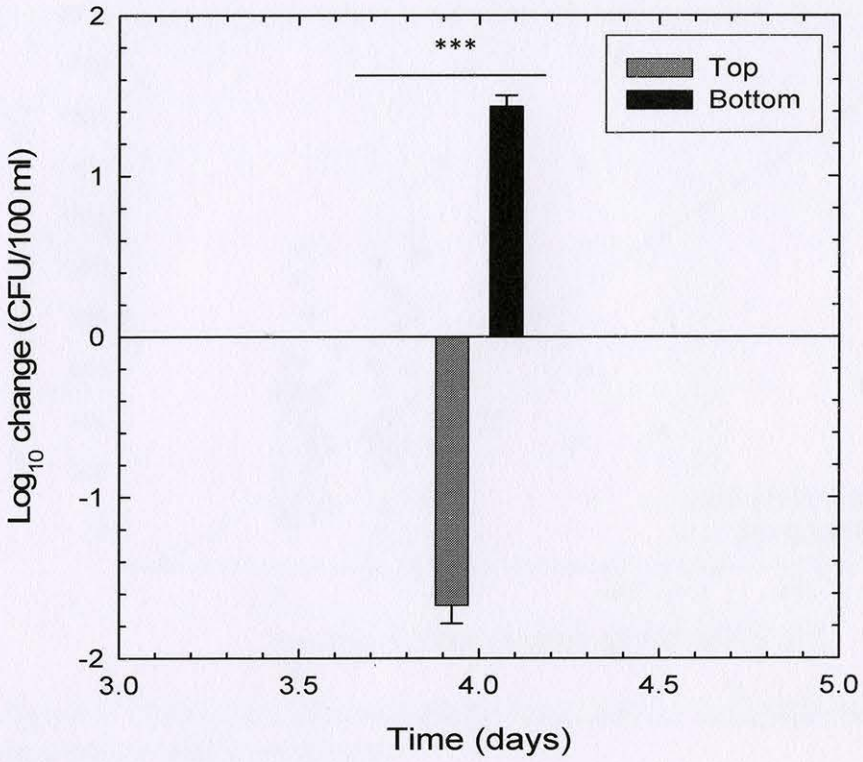

Figure 8. Effect of water column location on enterococci survival in environment waters. Each bar is the averaged Log_{10} change of ten microcosm replicates. *** denotes significant differences between the two locations with a P-value = 1.621×10^{-14}. Error bars represent standard error between the ten replicates.

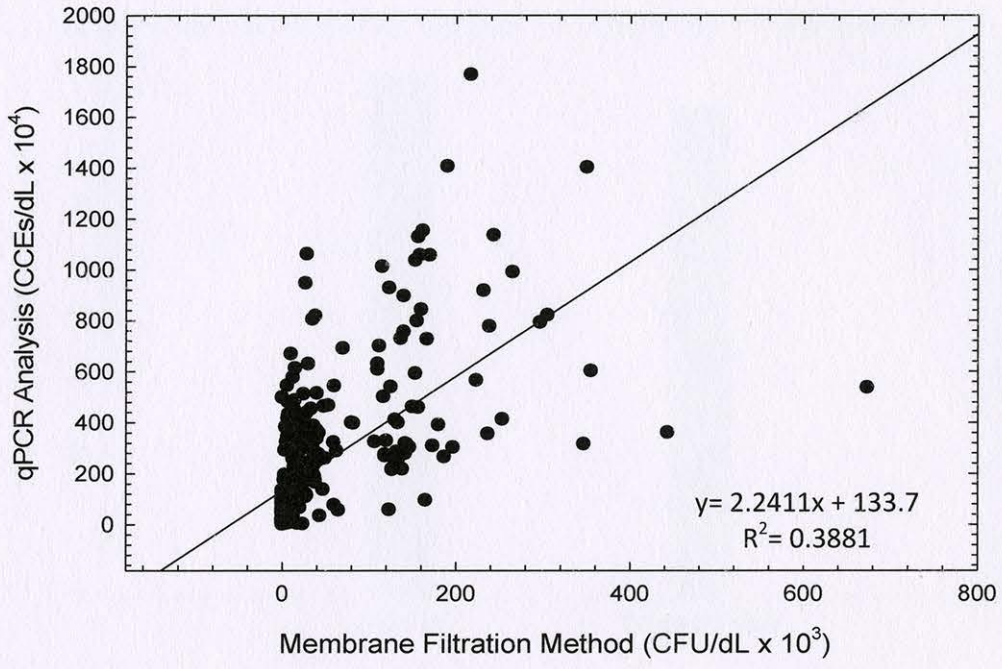

Figure 9. Comparison of membrane filtration method and qPCR analysis for enterococci enumeration and quantification.

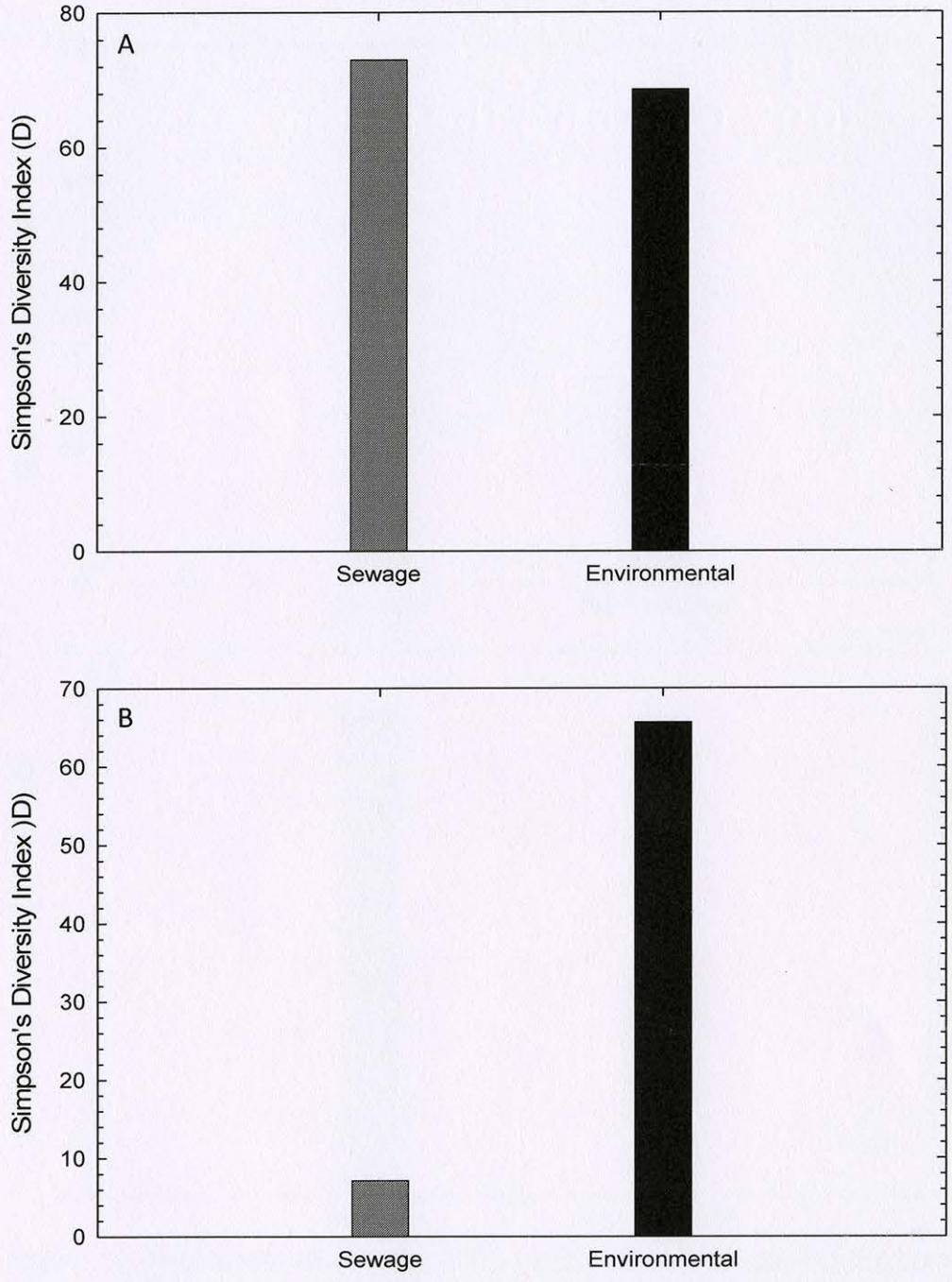

Figure 10. Comparison of the seasonal genetic diversity of sewage and environmental enterococci populations in A) summer and B) fall, using the Simpson's diversity index.

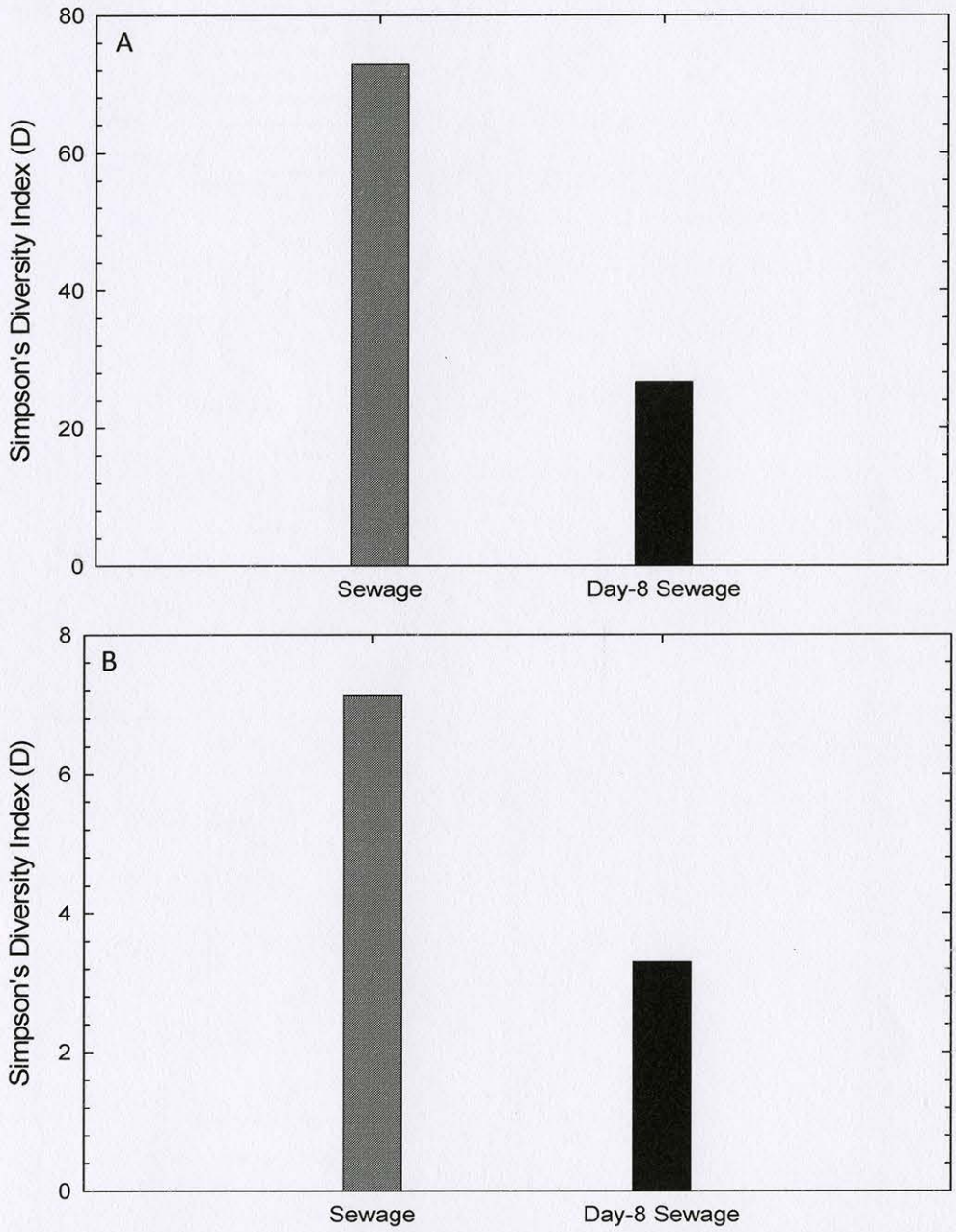

Figure 11. Assessment of selection in the environment by comparing the genetic diversity of sewage and Day-8 sewage enterococci populations in A) summer and B) fall, using the Simpson's diversity index.

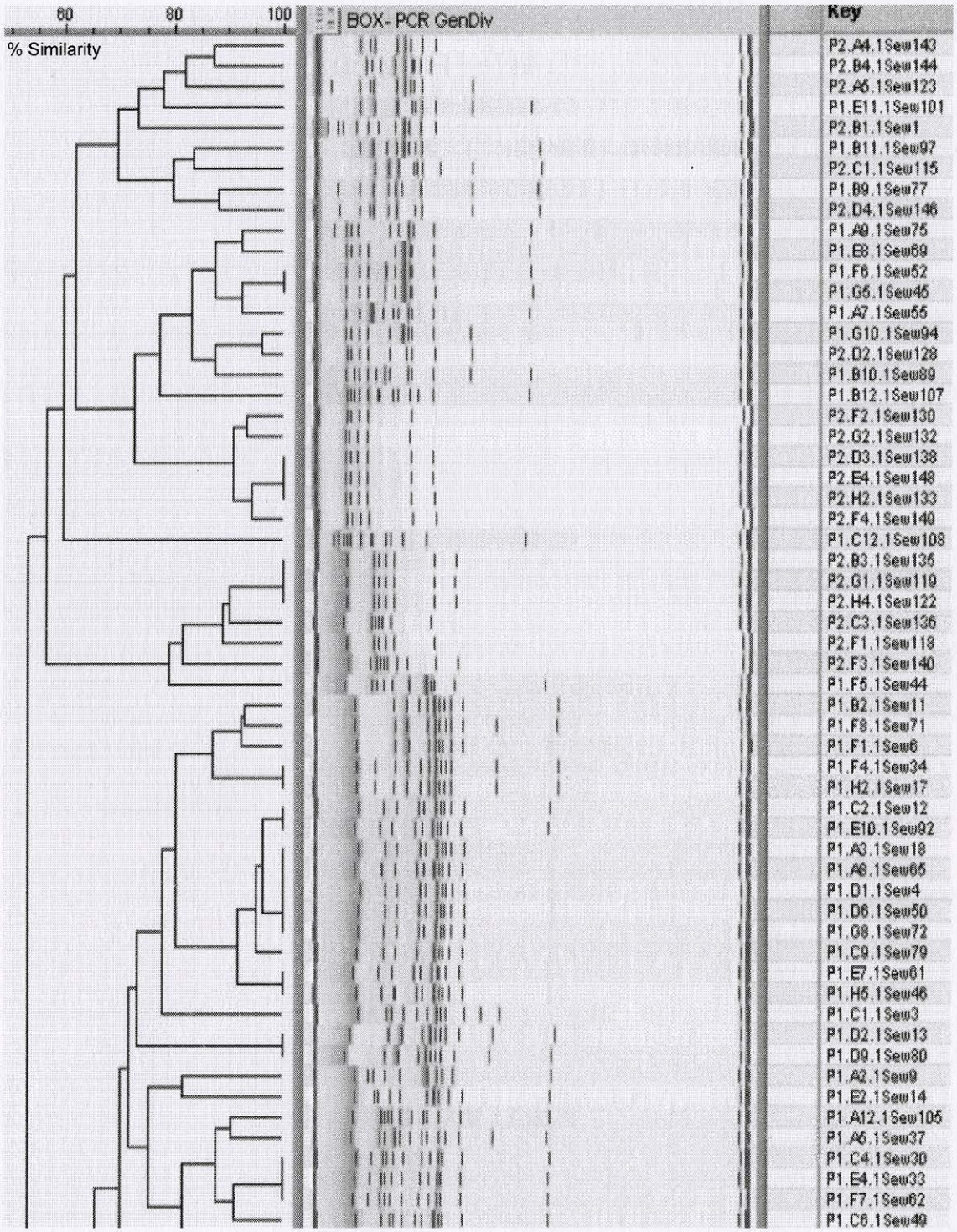

Figure 12. An example of the dendrograms used to compare fingerprints. Similarity plot based on BOX-PCR fingerprints of a sample of enterococci isolates. Groupings based on 100% similarity.

Hardiness of Sewage and Environmental Isolates

Sewage isolates were hardier to oxidative damage than environmental isolates. Day-8 sewage isolates were the hardiest with 86% of the isolates having optical densities >1.6 after 36 hours of 2.5 mM hydrogen peroxide exposure, thus showing tolerance to oxidative damage. Sewage isolates had 59% of the isolates showing tolerance. Environmental isolates were the least hardy to the oxidative damage as only 22% of the isolates had optical densities >1.6 (Table 2). All negative controls confirmed sterility and all positive controls had optical densities >1.6.

Table 2

Number of enterococci isolates that grew in the presence of 2.5 mM hydrogen peroxide after 36 hours incubation

	Tolerant Isolates (%)	Total Isolates
Sewage Isolates	136 (59)	229
Environmental Isolates	50 (22)	228
Day-8 Sewage Isolates	220 (86)	257

CHAPTER V

DISCUSSION

Enterococci are commensal bacteria that are found in the feces of humans and other warm-blooded animals and are therefore used as FIBs. However, their reliability as FIBs has been scrutinized as they have been shown to proliferate and persist in environmental waters under field simulated laboratory studies (Lleo et al., 2005; Pote et al., 2009). The primary objective of this study was to characterize the persistence of sewage enterococci in seawater in the natural environment.

Results from the five field experiments showed that sewage enterococci do not persist in the water column. However, a small percentage of sewage isolates was able to survive for eight days (0-0.5% of initial concentrations) in natural seawater. The results of the field experiments were similar to those of the control laboratory experiment, thus indicating that enterococci did not escape the microcosms, but died. The observed low survivability is contrary to the results of other researchers. Lleo et al. (2005), for example, observed 60-80% of enterococci, when incubated at room temperature or 4°C, in oligotrophic water, remained viable for at least two weeks. Similarly, Pote et al. (2009) showed that when enterococci were incubated in the water column at 20-25°C, their concentration remained stable for 60 days and they also retained their culturability. Results of Lleo et al. (2005) and Pote et al. (2009) may contradict results of the present study due to differences in the testing conditions. The previous studies were carried out at constant temperatures and conditions. The present study was conducted in the natural environment where conditions such as temperature, wind speed and direction, rainfall event, and salinity constantly vary and have been shown to impact FIB counts (Wymer et

al., 2005). These environmental conditions may change within a few minutes, hours, or daily. Therefore, the data of the present study more accurately represent how sewage enterococci survive in environmental waters.

The low survivability observed in the field experiments could have been caused by biotic and abiotic stressors. These stressors, for example oligotrophy, salinity, and predation, have all been shown to negatively impact enterococci survival in marine waters (Anderson et al., 2005; Bordalo, Onrassami, & Dechsakulwatana, 2002; Hartke, Lemarinier, Pichereau, & Auffray, 2002; Lleo et al., 2005; Menon et al., 2003). UV light was excluded as a stressor as enterococci were completely enclosed in the microcosms. The differential survival of sewage enterococci could be an indication that when they are released into the environment, they may in a weakened state and are thus more susceptible to the stressors of environmental marine waters. Therefore, only hardy isolates would survive.

QPCR data from the field experiments also revealed that enterococcal DNA rapidly declined. The rapid decline of enterococcal DNA supports the data from the culture method and is a clear indication that enterococci cells died when deployed in the water column. Studies have shown that enterococci tend to become VBNC when placed in stressful environments such as oligotrophic waters and salinity (Heim et al., 2002; Lleo et al., 2001; & Lleo et al., 2005). However, the results of the present study show that sewage enterococci, when released in environmental marine waters, do not become VBNC but instead exhibit high mortality.

Data also revealed a seasonal effect on the rate of decline of viable sewage enterococci and enterococcal DNA concentration. The rate of decline was higher for both

viable cells and DNA during summer months when the water temperature was highest, and lower during fall and winter, when water temperature was lowest. These results were similar to those observed by Howell, Coyne, and Cornelius (1996). A possible explanation could be the effect of enterophage activity. While the effect of phage on the persistence of enterococci was not tested in the present study, Santiago-Rodriguez et al. (2010) showed that enterophage survived longer at higher temperatures (37 and 41°C) than at a lower temperature (22°C) in seawater. Moldovan, Chapman-McQuistan, and Wu (2007) also showed that phage adsorption to bacteria increased as water temperature increased. The variation of temperature between seasons may have resulted in an increased affinity between the surface receptors of the bacteria and the phage during the summer but a reduced interaction during the fall and winter. In other words, more enterococci may have been lysed or killed by enterophage in the summer than they would in the fall or winter.

Statistical analysis of viable counts for the filtered and natural beach water samples showed a difference in enterococci concentration (Table 1). Predation effect varied between sites and was attributed to the high sewage concentration (1:1) used to inoculate the microcosms on Day 0. As a result of using a high sewage concentration, there was a low predator: prey ratio which in turn masked the effect of predation. Supporting data were shown in Figure 7 where the effect of predation was more obvious in the 1:100 dilution than the 1:1 dilution.

My results showed a positive correlation between the results obtained using the MF and qPCR methods. Similar data trends were reported by Haugland et al. (2005) and is an indication of the usefulness of the qPCR method, which is not only rapid (three to

four hours) but also sensitive. The level of correlation however, indicated that results obtained using the qPCR method cannot be used to directly quantify enterococci due to low confidence. While the MF method only enumerates viable enterococci, qPCR quantifies total DNA (live, dead, and VBNC cells and exogenous DNA). One useful solution would be the implementation of PMA (propidium monoazide) treatment of water sample before qPCR analysis (Walters, Yamahara, & Boehm, 2009). PMA would bind exogenous DNA and DNA from compromised cells, thus preventing them from being amplified.

The location of enterococci in the water column affects their survival rates. Enterococci had >25 fold increase in cell density when microcosms where placed at the bottom of the water column but >45 fold decrease when placed at the top (Figure 8). These results are an indication that there may be more nutrient in the water just above the bottom that is able to support bacterial growth. The nutrient gradient may be originating from the benthic sediment or from settled particulates such as detritus. These nutrients may be incorporated only into the bottom of the water column due to the low wave energy in the Mississippi Sound. The presence of a nutrient gradient may indicate that enterococci survival and persistence is dependent on their location in the water. The occurrence of a nutrient gradient has strong implications for water quality monitoring. Enterococci surviving at the bottom of the water column may be subjected to resuspension by storm activity, wave action, or water current.

The presence of a nutrient gradient in the water column may be another explanation for the rapid decline in enterococci concentration observed in the current persistence field study. In the current study, the microcosms were placed at the top of the

water column. Consequently, the rapid decline of enterococci concentration in the microcosms may have been caused by the oligotrophic condition of the surface water. The oligotrophic condition of the surface water may have also caused the high mortality observed in the field experiments.

The genetic diversity of the sewage population varied seasonally while that of the environmental population remained high. The seasonal variation of sewage isolates indicated that during the fall, the less hardy isolates died, resulting in a less genetically diverse population compared to that of summer. No seasonal effect was observed for the environmental population as it maintained a high genetic diversity. The genetic diversity results do not support my initial hypothesis. I hypothesized that only the hardy sewage enterococci isolates would survive in the environment, therefore resulting in a genetically less diverse environmental population compared to that of the sewage. However, the maintained diversity of the environmental population may have been caused by an accumulation, over time, of hardy isolates that were all able to tolerate environmental stress. These isolates are possibly being maintained in an environmental reservoir such as sand (Byappanahalli et al., 2006).

Day-8 sewage isolates were genetically less diverse than initial sewage isolates both in summer and winter months. The results indicated that sewage enterococci, when released in environmental waters, were exposed to selective pressures. The hardier isolates were selected for environmental survival and this resulted in a less genetically diverse Day-8 sewage population. The initial hypothesis that sewage enterococci would be selected in the environment is supported. Also, the claim in the previous paragraph

that the environmental enterococci population might be composed of an accumulation of hardy isolates was also supported by this data.

Enterococci found in sewage, were hardier against oxidative damage than those in beach water. Particularly, the Day-8 sewage isolates were the hardiest and environmental isolates were the least hardy. The initial hypothesis that enterococci found in beach water would be hardier than those in sewage is rejected. Instead, the lower tolerance of enterococci from beach water indicated that oxidative damage was not their main selective pressure. None the less, the increased tolerance of Day-8 sewage isolates is a clear indication that they were selected for survival in the environmental.

In conclusion, the goal of this study was to characterize the persistence of sewage enterococci in marine water, as well as to determine if the genetic diversity or hardiness differed between sewage and environmental enterococci. Results showed that sewage enterococci and their DNA do not persist in surface waters. However, their ability to persist in the environment seemed to depend on their location in the water, as the possible occurrence of a nutrient gradient may permit bacterial growth. A comprehensive analysis of surface and bottom environmental beach water must be untaken to more accurately and definitively characterize this potential nutrient gradient. Sewage enterococci were shown to differentially survive in the environment as Day-8 sewage isolates had a lowered genetic diversity. Conversely, enterococci in beach water maintained a high genetic diversity which indicated the accumulation of hardy isolates over time that may be maintained in an environmental reservoir. Also, Day-8 sewage isolates were the most tolerant to oxidative damage, confirming selection. Oxidative damage does not appear to be the main selective agent against enterococci survival in the beach environment.

REFERENCES

Aarestrup, F. M., Butaye, P., & Witte, W. (2002). Nonhuman Reservoirs of Enterococci. In M. S., Gilmore, D. B., Clewell, P., Courvalin, G. M.,Dunny, B. E.,Murray, & L. B., Rice (Eds.), *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance* (pp. 55-99). Washington, DC: ASM Press.

Anderson, K. L., Whitlock, J. E., & Harwood, V.J. (2005). Persistence and Differential Survival of Fecal Indicator Bacteria in Subtropical Waters and Sediments. *Applied and Environmental Microbiology, 71*(6), 3041-3048.

Badgley, B. D., Thomas, F. I. M, & Harwood, V. J. (2010). The Effect of Submerged Aquatic Vegetation on the Persistence of Environmental Populations of *Enterococcus spp. Environmental Microbiology, 12*(5), 1271-1281.

Barcina, I., Lebaron, P., & Vives-Rego, J. (1997). Survival of Allochthonous Bacteria in Aquatic Systems: A Biological Approach. *FEMS Microbiology Ecology, 23*, 1-9.

Boehm, A. B., Keymer, D. P., & Shellenbarger, G.G. (2005). An Analytical Model of Enterococci Inactivation, Grazing, and Transport in the Surf Zone of a Marine Beach. *Water Research, 39*(15), 3565-3578.

Bordalo, A. A., Onrassami, R., & Dechsakulwatana, C. (2002). Survival of Fecal Indicator Bacteria in Tropical Estuarine Waters (Banpakong River Thailand). *Journal of Applied Microbiology, 93*(5), 864-871.

Byappanahalli, M. N., Whitman, R. L., Shively, D. A., Ting, W. T., Tseng, C. C., & Nevers, M. B. (2006). Seasonal Persistence and Population Characteristics of *Escherichia coli* and Enterococci in Deep Backshore Sand of Two Freshwater Beaches. *Journal of Water and Health, 4*(3), 313-320.

Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R., & Harwood, V.J. (2012). Enterococci in the Environment. *Microbiology and Molecular Biology Reviews, 76*, 685-706.

Cabelli, V. J. (1983). *Health Effects Criteria for Marine Recreational Waters* (EPA 600/1-80-031). Research Triangle Park, N.C.

Dufour, A. P. (1984). *Health Effects Criteria for Fresh Recreational Waters* (EPA 600/1-84-004). Research Triangle Park, N.C.

Dufour, A., & Schaub, S. (2007). The Evolution of Water Quality Criteria in the United States, 1922–2003. In L. J., Wymer (Ed), *Statistical Framework for Recreational Water Quality Criteria and Monitoring* (pp.1-11). Hoboken, NJ: John Wiley and Son, Ltd.

Facklam, R. R., Carvalho M. G. S., & Teixeira L. M. (2002). History, Taxonomy, Biochemical Characteristics, and Antibiotic Susceptibility Testing of Enterococci. In M. S., Gilmore, D. B., Clewell, P., Courvalin, G. M., Dunny, B. E., Murray, & L. B., Rice, (Eds.), *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance* (pp. 1-54). Washington, DC: ASM Press.

Farrow, J. A. E., Jones, D., Phillips, B.A., & Collins, M.D. (1983). Taxonomic Studies on Some Group D Streptococci. *Journal of General Microbiology, 129*, 1423-1432.

Fisher, K., & Phillips, C. (2009). The Ecology, Epidemiology and Virulence of *Enterococcus*. *Microbiology, 155*, 1749-1757.

Flahaut, S., Hartke, A., Giard, J., & Auffray, Y. (1997). Alkaline Stress Response in *Enterococcus faecalis*: Adaptation, Cross-protection, and Changes in Protein Synthesis. *Applied and Environmental Microbiology, 63*(2), 812-814.

Flahaut, S., Laplace, J., Frere, J., & Auffray, Y. (1998). The Oxidative Stress Response in *Enterococcus faecalis*: Relationship Between H_2O_2 Tolerance and H_2O_2 Stress Proteins. *Letters in Applied Microbiology, 26*, 259-264.

Fujioka, R. S., Hashimoto, H. H., Siwak, E. B., & Young, R. H. F. (1981). Effect of Sunlight on Survival of Indicator Bacteria in Seawater. *Applied and Environmental Microbiology, 41*(3), 690-696.

Fujioka, R., Sian-Denton, C., Borja, M., Castro, J., & Morphew, K. (1999). Soil: The Environmental Source of *Escherichia coli* and Enterococci in Guam's Streams. *Journal of Applied Microbiology Symposium Supplement, 85*, 83S-89S.

Gonzalez, J. M., Iriberri, J., Egea, L., & Barcina, I. (1990). Differential Rates of Digestion of Bacteria by Freshwater and Marine Phagotrophic Protozoa. *Applied and Environmental Microbiology, 56*(6), 1851-1857.

Griffin, D. W., Lipp, E. K., McLaughlin, M. R., & Rose, J. B. (2001). Marine Recreation and Public Health Microbiology: Quest for the Ideal Indicator. *BioScience, 51*(10), 817- 826.

Gullberg, R. M. (1986). The Enterococcus. *Infection Control*, *7*(12), 600-606.

Hancock, L. E., & Gilmore, M. S. (2006). Pathogenicity of Enterococci. In V. A., Fischetti, R. P., Novick, J. J., Ferretti, D. A., Portnoy, & J. L., Rood (Eds.), *Gram-Positive Pathogens* (pp.299-311). Washington, DC: ASM Press.

Hartke, A., Giard, J., Laplace, J., & Auffray, Y. (1998). Survival of *Enterococcus faecalis* in an Oligotrophic Microcosm: Changes in Morphology, Development of General Stress Resistance, and Analysis of Protein Synthesis. *Applied and Environmental Microbiology, 64*(11), 4238-4245.

Hartke, A., Lemarinier, S., Pichereau, V., & Auffray, Y. (2002). Survival of Enterococcus faecalis in Seawater Microcosms is Limited in the Presence of Bacterivorous Zooflaggelates. *Current Microbiology, 44*(5), 329-335.

Haugland, R. A., Siefring, S. C., Wymer, L. J., Brenner, K. P., & Dufour, A. P. (2005). Comparison of Enterococci Measurements in Freshwater at Two Recreational Beaches by Quantitative Polymerase Chain Reaction and Membrane Filter Culture Analysis. *Water Research, 39*, 559-568.

Heim, S., Lleo, M., Bonato B., Guzman, C. A., & Canepari, P. (2002). The Viable but Nonculturable State and Starvation are Different Stress Responses of *Enterococcus faecalis*, as Determined by Proteome Analysis. *Journal of Bacteriology, 184*(23), 6739–6745.

Howell, J. M., Coyne, M. S., & Cornelius, P. L. (1996). Effect of Sediment Particle Size and Temperature on Fecal Bacteria Mortality Rates and the Fecal Coliform/Fecal Streptococci Ratio. *Journal of Environmental Quality, 25*, 1216-1220.

Lindell, S. S., & Quinn, P. (1975). Use of Bile-esculin Agar for Rapid Differentiation of Enterobacteriaceae. *Journal of Clinical Microbiology, 1*(5), 440-443.

Lleo, M.M., Bonato, B., Tafi, M. C., Signoretto, C., Boaretti, M., & Canepari, P. (2001) Resuscitation Rate in Different Enterococcal Species in the Viable but Non-culturable State. *Journal of Applied Microbiology, 91*(6), 1095-1102.

Lleo, M. M., Bonato, B., Benedetti, D., & Canepari, P. (2005). Survival of Enterococcal Species in Aquatic Environments. *FEMS Microbiology Ecology, 54*(2), 189-196.

Ludwig, W., & Schleifer, K. H. (2000). How Quantitative is Quantitative PCR with Respect to Cell Counts? *Systematic and Applied Microbiology, 23*(4), 556-562.

Manero, A., & Blanch, A. R. (1999). Identification of *Enterococcus spp.* with a Biochemical Key. *Applied and Environmental Microbiology, 65*(10), 4425-4430.

Menon, P., Billen, G., & Servais, P. (2003). Mortality Rates of Autochthonous and Fecal Bacteria in Natural Aquatic Ecosystems. *Water Research, 37*(17), 4151-4158.

Moellering, R. C. (1992). Emergence of Enterococci as a Significant Pathogen. *Clinical Infectious Disease, 14*(6), 1173-1176.

Moldovan, R., Chapman-McQuistan, E., & Wu, X. L. (2007). On Kinetics of Phage Adsorption. *Biophysical Journal, 93*(1), 303-315.

National Technical Advisory Committee. (1968). *Water Quality Criteria* p. 4. Washington, DC: U.S. Government Printing Office.

Pote, J., Haller, L., Kottelat, R., Sastre, V., Arpagaus, P., & Wildi, W. (2009) Persistence and Growth of Fecal Culturable Bacterial Indicators in Water Column and Sediment of Vidy Bay, Lake Geneva Switzerland. *Journal of Environmental Science (China), 21*(1), 62-69.

Rince, A., Breton, Y. L., Verneuil, N., Giard, J., Hartke, A., & Auffray, Y. (2003). Physiological and Molecular Aspects of Bile Salt Response in *Enterococcus faecalis*. *International Journal of Food Microbiology, 88*, 207-213.

Santiago-Rodriguez, T. M., Davila, C., Gonzalez, J., Bonilla, N., Marcos, P., Urdaneta, M.,… & Toranzos, G. A. (2010). *Water Research, 44*, 4716-4725.

Santo Domingo, J. W., Siefring, S. C., & Haugland, R. A. (2003). Real-time PCR Method to Detect *Enterococcus faecalis* in Water. *Biotechnology Letters, 25*, 261-265.

Scott, T. M., Rose, J. B., Jenkins, T. M., Farrah, S. R., & Lukasik, J. (2002). Microbial Source Tracking: Current Methodology and Future Directions. *Applied and Environmental Microbiology, 68*(12), 5796-5803.

Sherman, J. M. (1937). The Streptococci. *Bacteriology Rev*eiws, *1*(1),3-97.

Sherman, J. M . (1938). The Enterococci and Related Streptococci. *Journal of Bacteriology, 35*(2), 81-93.

Simons, G. W., Hilscher, R., Ferguson, H. F., & Gage, S. (1922). Report of the Committee on Bathing Places. *American Journal of Public Health, 12*(2), 121-123.

Simons, G. W., Gillespie, C. G., Gage, S., Ferguson, H., & Tisdale, E.S. (1924). Report of the Committee on Bathing Places. *American Journal of Public Health, 14*(7), 597-602.

Snyder, M. L., & Lichstein, H. C. (1940). Sodium Azide as an Inhibiting Substance for Gram-Negative Bacteria. *The Journal of Infectious Diseases, 67*(2), 113-115.

U.S. Environmental Protection Agency. (1986*). Quality Criteria for Water-1986* (EPA 440/5-86-001). U.S. Environmental Protection Agency, Office of Water, Washington, DC.

U.S. Environmental Protection Agency. (1992). *Summary of State Water Quality Recreational Standards for Bacteria*. U.S. Environmental Protection Agency, Office of Water Policy Analysis, Washington DC.

U.S. Environmental Protection Agency. (2000*). Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000* p. 871. Public Health and Safety. Washington, DC.

U.S. Environmental Protection Agency. (2002). *Method 1106.1: Enterococci in Water by Membrane Filtration using membrane-Enterococcus-Esculin iron Agar (mE-EIA)* (EPA-821-R-02-021). U.S. Environmental Protection Agency, Office of Water, Washington DC.

U.S. Environmental Protection Agency. (2003). *Bacterial Water Quality Standards for Recreational Waters (Freshwater and Marine Waters).* Status Report (EPA-823-R-03-008). U.S. Environmental Protection Agency, Washington, DC.

U.S. Environmental Protection Agency. (2006). *Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI)* (EPA-821-R-06-009). U.S. Environmental Protection Agency, Office of Water, Washington DC.

U.S. Environmental Protection Agency. (2012*). Method 1611: Enterococci in Water by TaqMan Quanititative Polymerase Chain Reaction (qPCR) Assay* (EPA-821-R-12-008). U.S. Environmental Protection Agency, Office of Water, Washington DC.

Walters, S.P., Yamahara, K.M., & Boehm, A.B. (2009). Persistence of Nucleic Acid Markers of Health-relevant Organisms in Seawater Microcosms: Implications for their use in Assessing Risk in Recreational Waters. *Water Research, 43*, 4929-4939.

Weaver, K. E. (2006). Enterococcal Genetics. In V. A., Fischetti, R. P., Novick, J. J., Ferretti, D. A., Portnoy, & J. I., Rood, (Eds), *Gram-Positive Pathogens* (pp. 312-331). Washington, DC: ASM Press.

Wilson, S.G., & Fischetti, T. R. (2010). *Coastline Population Trends in the United States: 1920 to 2008*. U.S. Census Bureau. U.S. Department of Commerce, Washington, DC.

Wymer, L. J., Nrenner, K.P., Martinson, J.W., Stutts, W.R., Schaub, S.A., & Dufour, A.P. (2005). *The EMPACT Beaches Project: Results from a Study on Microbiological Monitoring in Recreational Waters* (EPA 600/R-04/023). U.S. Environmental Protection Agency, Office of Research and Development, Cincinnati OH.

Yamahara, K. M., Walters, S. P., & Boehm, A. B. (2009). Growth of Enterococci in Unaltered, Unseeded Beach Sands Subjected to Tidal Wetting. *Applied and Environmental Microbiology, 75*(6), 1517-1524.